The energetics of peptide bond formation at elevated temperatures.

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Award date:
1976

Awarding institution:
University of Bath

THE ENERGETICS OF PEPTIDE BOND

FORMATION AT ELEVATED TEMPERATURES.

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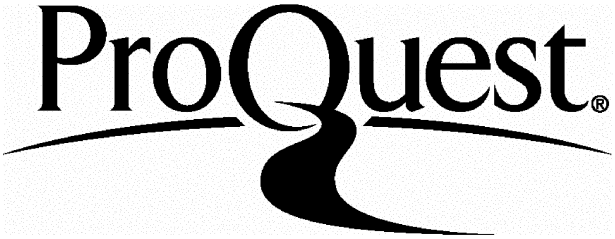
for the degree of Ph.D.

of the University of Bath

1976



ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. A.W. Flegmann for his invaluable advice, support, and encouragement throughout the course of this work.

I also wish to thank Professor A.H. Rose and Professor L. Broadbent for allowing me the use of School facilities to pursue this study. Grateful thanks also to Mrs. Judith Hunt for cheerfully and efficiently deciphering the manuscript and expertly typing the thesis.

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ABSTRACT

Published data on the energetics of peptide bond formation are scarce and virtually none have been reported at elevated temperatures. The energetics of the peptide bond forming reaction between the partially protected amino acids carbobenzoxyglycine and L-phenylalanine amide at a range of temperatures were therefore investigated in detail. The choice of reaction components ensured the unambiguous formation of only one type of peptide bond. The free energies at temperatures up to 60°C were determined experimentally by equilibrium measurements and up to 100°C by extrapolation. The peptide bond forming reaction in buffered solution at pH 7.2 was catalysed enzymatically. The isolation and testing of a thermophilic enzyme from Bacillus stearothermophilus is described. The enzyme finally selected as catalyst was thermolysin. The equilibrium constant measurements were made using radiotracers and the synthesis of ^{14}C labelled protected amino acids and dipeptides is described. An attempt was made to approach equilibrium from both directions. Ion-exchange chromatography and paper and thin layer chromatography and electrophoresis were tried for dipeptide isolation and purity assessment. Precipitation and crystallization methods were finally employed. Ionization constants were determined by potentiometry at temperatures up to 80°C. The ΔG^{o} results at 25°C agreed favourably with the only previously published value derived from an equilibrium measurement. Although the free energy change with increasing temperature was found not to be large, the standard free energy for peptide bond formation did appear to become negative at temperatures exceeding 60°C. The enzyme was found to affect the equilibrium at high dipeptide/enzyme

molar ratios. The enthalpy of peptide bond formation was calculated from the van't Hoff equation and the entropy subsequently derived.

CHAPTER I

A REVIEW OF PEPTIDE BOND FORMATION

1:1 INTRODUCTION

Prior to 1950 it was generally considered that peptide bonds could not be formed to any significant extent by the reversal of hydrolysis in aqueous solution in the range of physiological temperatures. This view was based on free energy data (1) (2) derived largely from calorimetric studies, which indicated that large gains in free energy, from +2 to +4 kilocalories, were required for the formation of a dipeptide from its constituent amino acids, and that under ordinary conditions spontaneous synthesis was negligible. Work by Fruton and his colleagues published in 1951 and 1952 (3) (4) employing, for the first time, equilibrium measurements to estimate the free energy of peptide bond hydrolysis, produced values of +520 and +420 calories per mole at 25°C for the peptide forming reaction. These figures represented a concentration of over 1% dipeptide at equilibrium indicating that during the condensation of some peptides, the free energy change may be small enough that, under certain conditions, a limited but measurable degree of synthesis can take place. Investigations by Wilson and Cannan (5) on the glutamic acid-pyrrolidonecarboxylic acid system had previously shown that at various extremes of pH and at temperatures of 78° to 118°C, the formation of an intramolecular amide bond was preferred. For example, at a temperature of 78°C and at a pH of 2.6, 86.3% of the glutamic acid condensed to form pyrrolidonecarboxylic acid. Calculations from this datum produced a value of -1290 calories per mole for the free energy change.

In view of these wide discrepancies in published data and the lack of information on peptide formation at high temperatures, this work was undertaken in order to provide values for the energetics of peptide bond formation at elevated temperatures.

The largest contribution to the free energy value of peptide bond formation in aqueous solution is not from the actual forming of the CO - NH linkage, but is due to the energy required to suppress the ionization of the reacting carboxyl and amino groups.

The formation of a peptide bond between two amino acids by classical chemical techniques involves the protection of the non-reacting carboxyl and amino groups to prevent side reactions, the suppression of ionization of the reacting carboxyl group by the introduction of an activating group, and the formation of a peptide bond between the two amino acids by the concurrent hydrolysis of an added condensing agent. Modern peptide synthetic methods have developed these techniques to the stage that the activation and condensation steps may be performed with one reagent in a single process, and protecting groups may be removed selectively from the amino or carboxyl groups without disruption of the peptide bond.

Peptides are synthesized biologically by the formation of the amino acyl-adenylates by amino acid specific enzymes. These extremely reactive compounds are stabilized by remaining associated with the parent enzyme and are transferred to a specific acceptor

molecule of s-RNA. A peptide bond is formed in the presence of a condensing enzyme by a nucleophilic attack of the α amino group of one amino - acyl s-RNA on the carboxyl carbon of a second amino - acyl s-RNA attached to m-RNA, with the subsequent release of the s-RNA, (6).

The reaction under consideration in this work, peptide bond formation by the reversal of hydrolysis, differs from these two procedures, as neither activating nor condensing agents are introduced to provide the energy required to form a bond. However it is analogous to 'prebiotic' synthesis.

Studies on the 'origin of life' have unequivocally demonstrated the formation of amino acids under simulated prebiotic conditions. The condensation of these molecules to form peptides and proteins is currently an area of intensive investigation. Recent reports (7) (8) (9) on the polymerization of amino acids in dilute aqueous solution at elevated temperatures on clay templates has been considered a possible prebiotic process, but the mechanism of this polymerization is obscure.

It was hoped that this work would help to evaluate the contribution of temperature to amino acid condensation in aqueous solution, and, in conjunction with current theories of peptide formation under prebiotic conditions, clarify possible primitive earth polymerization mechanisms. In order to assess possible mechanisms it is necessary to review the current theories and experimental evidence for the

evolution of life on earth.

1:2 PREBIOTIC PEPTIDE-BOND FORMATION

A prerequisite to the theories of peptide bond formation on the primitive earth is the formation of the earth itself and the conditions prevailing in such an abiotic environment.

Current theory suggests that the Earth and solar system were formed some 4.6×10^9 years ago (10) from the condensation of a cloud of swirling cosmic dust. Heating and aggregation of this dust eventually led to the formation of the planets and sun. The molten mixture of elements which formed Earth gradually cooled and at a period of about 3.6×10^9 years ago had produced conditions conducive to the synthesis of organic molecules.

The elements for primordial organic synthesis came from the primitive atmosphere, hydrosphere and lithosphere.

The primitive atmosphere probably evolved from volcanic outgassings and consisted mainly of water vapour, carbon dioxide, nitrogen and, to a lesser extent, methane, ammonia, carbon monoxide and hydrogen. This reducing atmosphere would have favoured the spontaneous generation of organic matter and conferred a stability on the molecules formed through lack of oxidation.

The primitive hydrosphere was of prime importance in the evolutionary sequence since 'life' is dependant upon water.

Evidence (11) suggests that the oceans have changed very little in salinity and pH since Precambrian times. Weathering of the primitive lithosphere would have provided ample supplies of clays and silts whose buffering action and ion-exchange properties would have maintained the pH of the primitive oceans somewhere around pH 8.0. The accumulation of biochemical compounds in these primitive waters, is, however, the basis of much current debate.

The temperature of the surface of the earth and oceans in earlier epochs remains unsolved. Geological evidence indicates that surface temperatures have not changed significantly during the last several hundred million years suggesting that in prebiotic times it was most likely around or below 100°C. This hypothesis has been lent credence by Hoyle (12) who has calculated that 3×10^9 years ago the increased intensity of sunlight striking the earth would have maintained a sea - level temperature of around 100°C.

The constituents of the primitive lithosphere, hydrosphere and atmosphere contained the elements necessary for the evolution of organic molecules. The energy required for the formation and reaction of these molecules could have been from several sources.

The sun would have been the most abundant form of energy on the primitive earth. However only 1% of its emitted radiation is at wavelengths low enough to be absorbed by organic precursors such as methane, water and ammonia,and so was probably ineffective in the synthesis of organic compounds. Nevertheless, ultra violet

and visible light inducing photochemical transformations in atmospheric and oceanic environments, the accumulation and concentration of products by the evaporation of water, and the polymerization of micro-molecules in heated pools and oceans may all have been important solar contributions.

The most significant contribution to the synthesis of organic molecules, such as amino acids, is considered to have been played by electric discharges. Lightning and corona discharges are able to generate electrons with sufficient energies to ionize and electrically excite molecules and promote 'micromolecular' synthesis.

Thermal energy from the earth's interior was one of the few forms of energy available in sufficiently small quanta to bring about specific interaction of molecules without undue destruction. This energy, in the form of volcanic activity and hot springs and pools, was probably particularly important in the condenstation of monomers to form polymers.

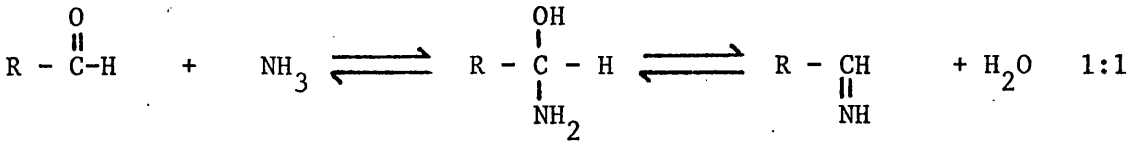
Other forms of energy, such as cosmic rays, radioactive disintegration and pressure forces are considered to have contributed to a minor extent to amino acid syntheses and polymerizations on the primitive earth.

Evidence to support these hypothetical primordial modes of molecular evolution has been presented within the last 25 years.

The classic work of Miller (13) (14) (15) (16) in the 1950's was the first unequivocal evidence of amino acid synthesis from simple organic precursors under simulated primitive earth conditions. In his first experiments he exposed gaseous mixtures of methane, ammonia, water and hydrogen to electric discharges in an apparatus designed to circulate the gases past the electrodes and condense the discharge products into a flask half filled with boiling water. The experiments were generally run for one week after which the gases were pumped out and the accumulated products analysed chromatographically and by mixed melting point techniques. Over twenty compounds were identified, representing 15% of the carbon added to the apparatus, including many amino acids, simple organic acids and cyanide derivatives. Subsequent experiments by various workers (See (17)) using different energy sources, such as silent discharges or ultraviolet light, and different reducing mixtures incorporating gases such as carbon monoxide and nitrogen, have confirmed that most aliphatic and aromatic amino acids, as well as various biologically significant unsaturated compounds, are formed. All the common amino acids with the exception of histidine have been synthesized under prebiotic conditions (17).

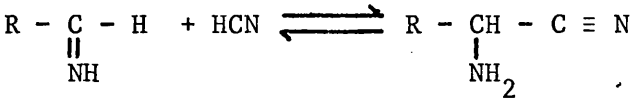
The mechanisms of prebiotic amino acid formation are divided into two main synthetic routes:

The Strecker synthesis involves the addition of ammonia to an aldehyde to form an imine.

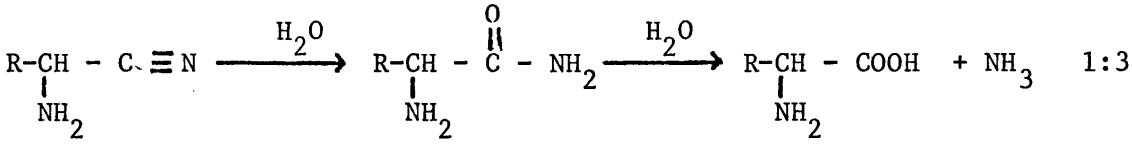


Simple aliphatic aldehydes, e.g. formaldehyde, acetaldehyde, are formed by the action of electric discharges or ultra-violet light on methane- water mixtures. Cyanide is also formed by this process.

The imine then reacts with cyanide to form an aminonitrile.

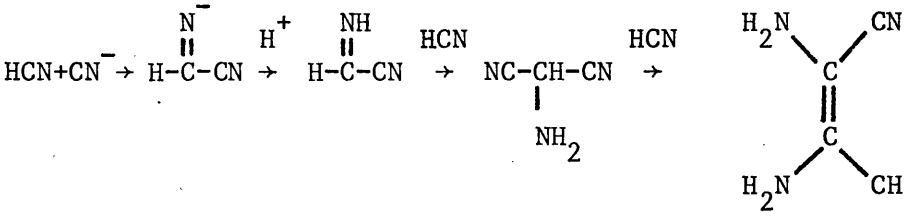


The reaction is completed by the irreversible hydrolysis of the nitrile to amino acid:



A parallel series of reactions is the addition of cyanide to an aldehyde to form a hydroxynitrile and its subsequent hydrolysis to form hydroxy acids. More complicated amino acids may be synthesized by variations on the Strecker synthesis, for example, the formation of acrolein from formaldehyde and acetaldehyde with subsequent addition reactions can form methionine or glutamic acid, (17),(18).

Amino acids may also be formed by hydrogen cyanide polymerization. In concentrated solution in the electric discharge apparatus, hydrogen cyanide undergoes a polymerization sequence forming a dimer, trimer and tetramer.



Trimer Tetramer 1:4

The latter two compounds may hydrolyse to form glycine, and the tetramer may initiate further polymerizations to form a heterogenous brown polymer which on hydrolysis yields a series of α amino acids (19).

Amino acids have been synthesized in low yield by heating methane, ammonia and water to about 1,000°C in silica tubes (20). Twelve common amino acids were identified among the products along with several non-protein amino acids. The major products of this pyrolysis were hydrogen cyanide and acetylene suggesting that the Strecker-type synthesis was probably the prominent reaction mechanism.

Various other forms of energy, ultraviolet light, ionizing radiations and shock waves, have all been tried as simulated prebiotic amino acid synthesizing systems with limited success.

The production of amino acids from reactive gases in the laboratory under a variety of conditions suggests that their production on the primitive earth was abundant. The preparation of peptides under prebiotic conditions is more problematical, not only from the adverse free energy point of view but also from the fact that the violent forms of energy required to synthesize the majority of amino acids leads to the breakdown of amino acid polymers. Energy in small quanta is required for peptide production. A variety of possible prebiotic peptide forming reactions have been demonstrated that shift the equilibrium in favour of amino acid condensation by either the removal of products from the site of reaction, the coupling of the reaction to the concurrent hydrolysis of another compound or the formation of peptides from active precursors.

The products of amino acid condensation are peptides and water. The removal of water from the site of reaction is most readily accomplished by heating. The thermal synthesis of polypeptides under anhydrous conditions has been extensively studied, particularly by S.W. Fox. Heating a mixture of anhydrous amino acids, always with equimolar or excess aspartic acid, glutamic acid or lysine to decrease the tendency of amino acid breakdown (21), to temperatures of 180^{o}C for several hours resulted in the formation of an amber glassy product. This product, in 10% to 40% yield, was termed ' a proteinoid' by Fox. Upon analysis proteinoids yield amino acids by acid hydrolysis, possess qualitative properties resembling those of

proteins and have a range of molecular weights (22). It was also reported that proteinoids had catalytic activity (23). The types of polymer linkage are still not fully resolved since prolonged hydrolysis has failed to give complete amino acid recovery in several cases (24)(25) suggesting that although most of the bonds in the thermal polypeptide are normal, α- β and α - γ amide links with aspartic and glutamic acid residues and abnormal bonds to the ε-amino group of lysine occur (25). It has been claimed (24) that the sequence of amino acids in the proteinoid does not appear random and that the order is self-determining being a function of the reacting amino acids. This point is still strongly disputed (26). The proteinoid , when heated with water and allowed to cool, deposits large numbers of microscopic globules, termed proteinoid microspheres by Fox, which have been claimed to possess a cellular type ultrastructure, with double layers, the ability to grow and proliferate and the ability to bind and retain macromolecules selectively (23). As Miller has pointed out (26) similar behaviour in other colloidal systems has been recorded.

The relevance of thermal polymerization as a prebiotic process is still the basis of much controversy. Fox has argued that geological anhydrizing temperatures would have been widespread on the primitive earth and sporadic rainstorms would have brought the intrusion of water necessary for microsphere formation (23). Miller, however, has maintained that 180^{o}C temperture sources around volcanic regions would have been few and probably beneath the la val surface making them inaccessible for amino acid polymerization, (26).

He favours more moderate temperatures for the thermal synthesis of protein.

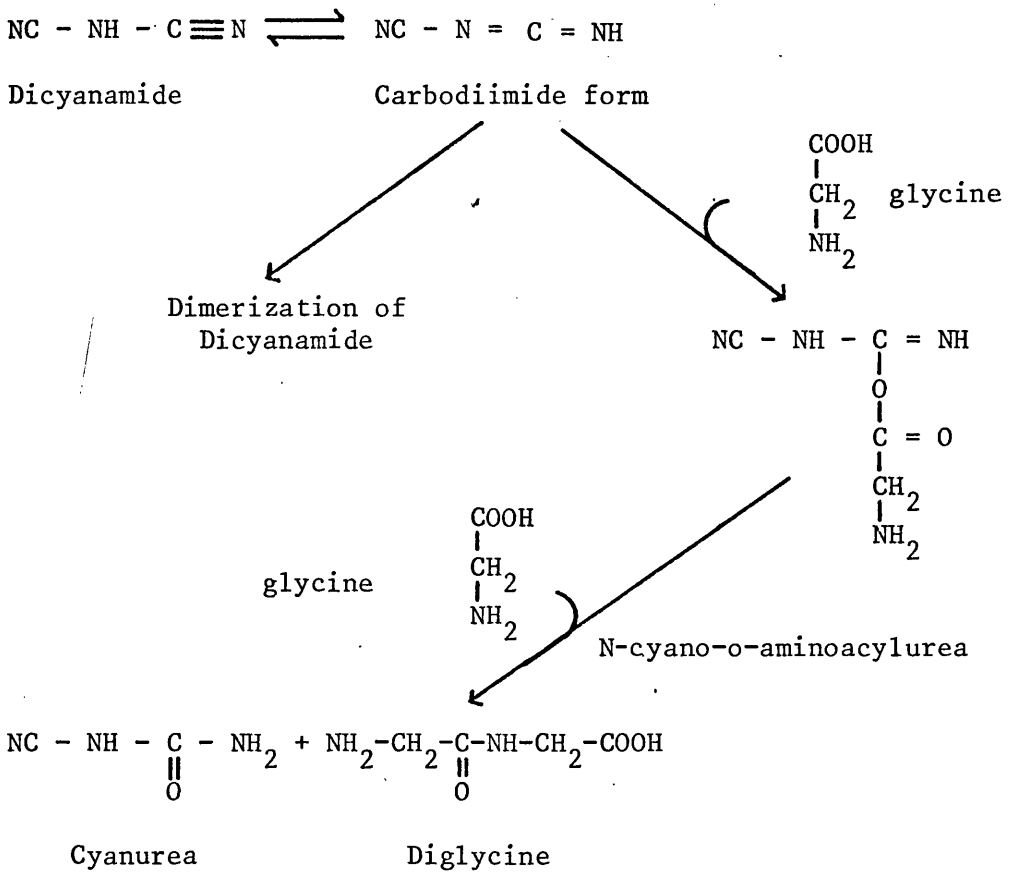
The condensation of amino acids in dilute aqueous solution and the removal of water from the site of reaction by evaporation is a second process which favours peptide formation. Bahadur and Ranganayaki (27) demonstrated that exposure of a dilute aqueous solution of glycine and sucrose to sunlight for one month resulted in the formation of several dipeptides, and it was suggested that the ultraviolet light was sufficiently energetic to accelerate this approach to thermodynamic equilibrium. Although the dipeptides were in very small concentrations it is likely that such syntheses occurred in the primitive oceans, lakes and pools. The synthesis in lakes and pools would have been particularly favoured by the evaporation of water from the surface, concentrating the polymerized products until even precipitation occurred.

The concentration of dilute peptide solutions could also have occurred by the adsorption onto surfaces, such as clays (28). This would have the effect of shifting the reaction in favour of peptide synthesis by removing the polymer product from solution. This concept was extended by Degens and Matheja (29) who demonstrated that kaolinite and montmorillonite clays adsorb amino acids onto their surfaces and at elevated temperatures promote their polymerization. Jackson (30) substantiated these claims and presented evidence for the selective adsorption and polymerization of L isomers of amino acids on the clay surface. These catalytic properties of clays are still under current investigation (31).

An alternative method of amino acid polymerization is the coupling of peptide bond condensation to the hydrolysis of energy rich compounds. The reaction employs simple stable reactants at moderate temperatures in an aqueous environment. It was shown by Lowe *et al.* (19) that heating an aqueous mixture of ammonium cyanide produced both amino acids and peptides. Miller had previously demonstrated that cyanides were formed from gaseous mixtures in his sparking experiments. (15). The possibility of using substituted cyanides as condensing agents was investigated by Steinman *et al.* (32) and Ponnamperuma and Peterson (33) who found that the cyanamides were suitable compounds. Cyanamide, which can tautomerize to carbodiimide and dimerize in dilute aqueous solution to dicyandiamide, was shown to promote peptide bond formation (32) (33), but only in low yields. Dicyanamide, however, was found to promote amino acid condensation in good yield and was investigated in detail by Steinman, Kenyon and Calvin (34), who suggested the following mechanisms for diglycine formation: Fig. 1:1.

The reaction occurs optimally at pH 2, hydrogen ions being required to protonate both the dicyanamide anion which would otherwise form, and the amino acid carboxyl group. The addition of the amino acid carboxyl group to the carbodiimide to form a cyano-o-aminoacylurea has to compete againstan amine catalysed dimerization of dicyanamide. This competition may be overcome by the slow addition of the condensing agent to the reaction mixture. Diglycine is formed by nucleophilic attack by a second

Fig 1:1



glycine molecule on the carbonyl group of the cyano-o-aminoacylurea with the liberation of cyanurea.

The synthesis of mixed peptides in solution occurs by a similar mechanism and Steinman (35) has presented evidence to indicate that the sequence of amino acids in the peptides formed is not random but self-determining based on the reactivity of component amino acids and the environment and pH of the system.

The formation of cyanamides by ultraviolet and sparking

experiments has been demonstrated (36), and their slow addition to amino acid solutions by washing from the site of synthesis in the primitive upper atmosphere by rainwater suggested (37). The presence of acidic conditions upon the primitive earth is unlikely even in localized volcanic areas but Steinman *et al.* have reported dicyanamide mediated dipeptide synthesis at neutral pH (34) and have suggested that given sufficient time substantial yields of peptides would result.

The synthesis of peptides from activated precursors has been suggested as a possible prebiotic condensation process. A novel synthesis of polypeptides from aminoacyladenylates on montmorillinite clay was reported by Paecht-Horowitz, Berger and Katchalsky (38). These authors reacted alanyladenylates with clay to produce polymers up to fifty residues long at almost 100% efficiency. However a suitable prebiotic synthesis of the parent adenylates has not yet been discovered.

It is the purpose of this work to determine the free energy of peptide bond formation at elevated temperatures and clarify the contribution of thermal energy to peptide formation in dilute aqueous solution.

1:3 THE ANALYSIS OF EXISTING THERMODYNAMIC DATA

Thermodynamic data for the heats of peptide bond formation have been derived using two techniques; calorimetry or equilibrium measurements. The majority of data has been obtained from

following the hydrolysis reaction.

There are two main calorimetric techniques that are of biochemical importance.

Combustion calorimetry is the classical and most general method of measuring heats of formation of organic substances. The heats of combustion of the reactants and products are determined in a bomb calorimeter, the heat of formation being equal to the sum of the heats of combustion of the products less the corresponding sum for the reactants. The resulting enthalpy change refers to a hypothetical process involving reactants and products in their standard states, usually pure solid or liquid compounds. Combustion calorimetry, as a method of studying biochemical reactions has several limitations. The difference in heat content between reactants and products is relatively small and calls for a high degree of precision in measurement. The data sought pertain to reactions occurring in solution under specified conditions and so enthalpy changes estimated from heats of combustion must be corrected by means of additional thermodynamic data on heats of solvation, ionization etc. Nevertheless the advantages of combustion calorimetry are that it is not restricted to perfectly thermodynamically reversible systems, it uses pure chemicals enabling precise definition of reaction conditions and, as a result, it provides the most accurate and reliable enthalpy data on pure materials. However reaction calorimetry has largely replaced combustion calorimetry in the biochemical field.

Reaction calorimetry involves the direct determination of heats of reaction. The reactants are placed together in a calorimeter with a suitable catalyst, such as an enzyme, and the temperature change in the reaction vessel recorded as a function of time as the reaction proceeds. The application of reaction calorimetry to biochemical reactions requires consideration of the stability of reactants and products and of the lack of adequate definition of initial and final states. Slow reactions are difficult to measure since any heat change is very small, and heats of mixing and diluting have to be allowed for in computations of final reaction heats. However the advantage of reaction calorimetry lies in the fact that measurements are carried out under conditions relatively close to those obtaining in a biological system.

Equilibrium studies are the alternative technique used to determine thermodynamic data. Equilibrium may be defined by considering the reversible reaction:

$$A + B \rightleftharpoons C + D$$

According to the Mass Action Law the rate of the forward reaction is k1 (A) (B) where k1 is a constant, and the rate of the reverse reaction is k2 (C) (D). At equilibrium the rates of forward and reverse reaction are equal and so k1 (A) (B) equals k2 (C)(D). If the ratio k1/k2 is set equal to a constant K, then:

$$\frac{(C)\ (D)}{(A)\ (B)} = K$$

where K is the equilibrium constant of the reversible reaction.

The equilibrium constant is related to both the enthalpy and free energy changes of a reaction.

The free energy change may be calculated directly from the equilibrium constant from the relationship

$$\Delta G^{o} = -RT \ln K$$

where ΔG^{o} is the standard free energy change, R is the gas constant, T the absolute temperature and ln K the natural logarithm of the equilibrium constant.

The enthalpy change may be calculated from the change in the equilibrium constant as a function of temperature by the van't Hoff equation:

$$\frac{d \ln K}{dT} = \frac{\Delta H^{o}}{RT^{2}}$$

where K is the equilibrium constant, T the absolute temperature, R the gas constant and ΔH^{o} the standard enthalpy change.

Since the equilibrium between amino acids and peptides lies on the far side of hydrolysis, sensitive methods for the estimation of product and reactant concentrations have to be employed to determine the equilibrium constant. The determination

of the constant also requires that equilibrium be reached as soon as possible under controlled conditions. This requires the use of a catalyst in the reaction system, such as an acid or base, or more frequently an enzyme. Once equilibrium has been reached the components of the reaction system must be separated and their concentrations measured. Provided a good catalyst can be found and a quantitative method of estimating reactant and product concentrations employed, equilibrium studies can provide valuable data on reaction thermodynamics.

A considerable amount of thermodynamic data have been accumulated on the hydrolysis and formation of peptide bonds. Some of this material has been collected and presented in Table 1:1.

Several points arise from the data collected in Table 1:1.

There is considerable variation between values for the heats of hydrolysis (ΔH^{o}) of the peptide bond at 25°C. The values obtained by combustion calorimetry are considerably higher than those obtained by reaction calorimetry. This is not surprising if account is taken of the fact that combustion calorimetry results have been calculated for amino acids and peptides in their standard states; in the case of Group 2 (Nos. 11 to 16) as solids. Reaction calorimetry values are all for reactions in solution. Variation between enthalpy

Table 1:1 THERMODYNAMIC DATA ON PEPTIDE BOND HYDROLYSIS AND SYNTHESIS

No	Substrate	Bond Hydrolysed	ΔH^o cal/mole	ΔG^o cal/mole	ΔS^o cal/mole/deg	Method	Reference
1	Poly lysine	lys-lys	-1,240	-	-	R	39
2	Bzl tyr-gly	tyr-gly	-1,330 ± 90	-	-	R	40
3	Tyr-gly NH_2	tyr-gly	-1,300 ± 150	-	-	R	40
4	Bzl tyr-glyNH_2	tyr-gly	-1,550 ± 100	-420 ± 50	-3.8 ± 0.4	R & E	4
5	Bzl-tyr	Bz acid-tyr	-1,980 ± 100	-	-	R	40
6	Bzl tyr-gly NH_2	Bz acid-tyr	-2,230 ± 210	-	-	R	40
7	CBZ gly-leu	gly-leu	-2,110 ± 50	-	-	R	41
8	CBZ gly-phe	gly-phe	-2,550 ± 50	-	-	R	42
9	Bzl-tyr NH_2	tyr-NH_2	-5,840 ± 220	-	-	R	42
10	Gly-phe NH_2	phe-NH_2	-6,220 ± 150	-	-	R	41
11	Gly-gly	gly-gly	-6,220 ± 620	-3,270 ±390	-9.9 ± 0.8	C	43
12	Ala-gly	ala-gly	-6,820 ± 620	-3,840 ± 390	-10.0 ± 0.8	C	43
13	Leu-gly	leu-gly	-5,590 ± 620	-3,120 ± 390	-8.3 ± 0.8	C	43
14	Hipp-gly	Hipp-gly	-4,980 ± 620	-2,420 ± 390	-8.6 ± 0.8	C	43

Table 1:1 (contd..)

15	Gly-phe	gly-phe	-6,100 ± 700	-	-	C	43
16	Ala-phe	ala-phe	-7,200 ± 700	-	-	C	43
17	Ala-gly (37.5°C)	ala-gly	-	-4,130	-	C	2
18	Gly-gly (37.5°C)	gly-gly	-	-3,590	-	C	2
19	Leu-gly (37.5°C)	leu-gly	-	-3,315	-	C	2
20	Hipp (37.5°C)	Bzl-gly	-	-2,650	-	C	2
21	Bzl gly-gly	Bzl-gly	-	-1,100	-	C	2
22	Gly-gly Ala-gly Leu-gly	AA_1 - AA_2	-	-3,380 ± 640	-	C	44
23	Ala-gly	ala-gly	-	-3,730	-	C	45
24	Gly-gly	gly-gly	-	-3,230	-	C	45
25	Hipp-gly	Hipp-gly	-	-2,380	-	C	45
26	Leu-gly	leu-gly	-	-2,960	-	C	45

Table 1:1 (contd..)

No	Substrate	Bond synthesized	ΔH^o	ΔG^o	ΔS^o	Method	Reference
27	CBZ gly + An CBZ gly + phz (40^oC)	gly-an gly-phz	- -	- -	- -	80% yield	46
28	HAc + Bu NH_2	Ac -buNH_2	+3,000	-	-	C	47
29	Pyrol/Glu (78^oC)	Pyrol/Glu	+3,600 + 500 + 3,800	+642 -1,290 +371	+ 8.4 - 2.2 +9.8	pH 1 2.6 12 } E	5

Table 1:1 ∿ All data at 25^oC unless otherwise stated

∿ See appendix for abbreviations

R = Reaction calorimetry

C = Combustion calorimetry

E = Equilibrium measurements

Group 1 ∿ Nos. 2 to 10 inclusive

Group 2 ∿ Nos. 11 to 16 inclusive

Group 3 ∿ Nos. 17 to 21 inclusive

results obtained by reaction calorimetry is interesting. Considering Group 1 (Nos 2 to 10), it has been suggested that the presence within a few Angström units of a positive charge on the carboxyl side or a negative charge on the amino side of a peptide bond, as in benzoyltryrosylglycine (No. 2), tyrosylglycinamide (No. 3), as compared with benzoyltyrosyl-glycinamide (No. 4), and the benzoic acid-tyrosine bond in benzoyltyrosine (No. 5) as compared with that in benzoyltyrosylglycinamde (No. 6), decreases the heat of hydrolysis by approximately 250 calories per mole. This is further illustrated by the value for polylysine hydrolysis (No. 1) where the formation of the peptide bond abolishes any charges present. The large difference in enthalpy values between peptides and amides is only partly attributable to the greater heat of ionization of the ammonium ion as compared to the substituted ammonium ions. The remaining difference in the heats of hydrolysis of the peptides and amides to form uncharged products is suggested as being largely due to the greater interaction of ammonia with the solvent. The values for the hydrolysis of CBZ glycylphenylalanine (No. 8) and the formation of n-butyl acetamide (No. 28) are higher, probably because the reactions were calculated to yield charged products whereas the others were not.

The values for the free energy of hydrolysis of the peptide bond do not differ as markedly between groups of results as within them. It appears that the configuration near a peptide bond influences the free energy of formation of that bond. For

instance, in Group 3 (Nos. 17 to 21) in order to form a dipeptide from two zwitterions (Nos. 17, 18, 19) more free energy is required than when one of the reactants is a simple ion (No. 21). When the charges not involved in the formation of the peptide bond are further apart than in an amino acid, the free energy change is again less, as in the case of hippurate (No. 20) compared with benzoylglycylglycine (No. 21). Continuing this process, when neither reactant is a zwitterion, when one is a cation and the other an anion, and both charges are abolished in the formation of the bond, the free energy change appears to be still less, as illustrated by benzoyltyrosylglycinamide (No. 4). However, the relatively low negative value for the free energy of hydrolysis of benzoyltyrosylglycinamide (No. 4) is striking, particularly as it was determined by equilibrium studies.

Also of particular note are the results of Wilson and Cannan (5) who demonstrated that the formation of the pyrrolidone-carboxylic acid ring from a glutamic acid molecule was a freely reversible reaction, the position of equilibrium being determined by the pH of the system. The enthalpy and free energy changes associated with such an intramolecular peptide bonding system were calculated from Wilson and Cannan's data as being comparatively large (No. 29).

It was hoped that the work undertaken here would clarify the apparently more favourable free energy data as measured

under equilibrium conditions and would determine the contribution of temperature to the free energy of peptide bond formation.

CHAPTER 2

ENZYME CATALYSIS - THE ISOLATION AND PROPERTIES OF THERMOPHILIC ENZYMES

2:1 ENZYME CATALYSIS IN EQUILIBRIUM STUDIES

(a) Enzymes as Perfect Catalysts

The rate at which a reversible chemical reaction reaches completion is governed by the rates of the forward and reverse reactions. When the two reaction rates are equal the system is said to be at equilibrium. In many reactions the rate of attainment of equilibrium is very slow unless a catalyst is used to increase the rate of reaction. One of the most versatile types of catalyst employed in chemical reactions are enzymes. The majority of enzymes are highly specific in the reactions they catalyse and increase the rate of both the forward and reverse processes.

The criteria for a perfect catalyst may be demonstrated by considering the reaction:

$$A + B + E_1 \rightleftharpoons C + D + E_2$$

where A and B are the reactants, C and D are the products and E_1 and E_2 the enzyme catalyst before and after the reaction.
At equilibrium:

$$\frac{(C)(D)(E_2)}{(A)(B)(E_1)} = \text{Constant K.}$$

If the enzyme operates as a perfect catalyst, E_1 equals E_2, the ratio E_2/E_1 equals 1, and hence the value of K is independent of enzyme concentration.

There are difficulties in testing whether an enzyme acts as a perfect catalyst since the quantity of enzyme used to catalyse a reaction is very small compared to substrate levels and, as most enzyme reactions proceed to practical completion under the conditions in which they can be best measured, the detection or isolation of different forms of enzyme would be very difficult. In even the best enzyme preparations the influence of slight contamination may cause some variation in the value of K. Several checks may be applied to indicate whether an enzyme acts as a perfect catalyst. Comparison of the equilibrium constant calculated from an enzyme catalysed reaction with that of the same reaction catalysed by another catalyst would indicate perfect catalysis if the two results were the same. The comparison of equilibrium constants from a chemical reaction catalysed by the same, or similar enzymes, prepared from different sources can also be used to indicate the degree of catalytic perfection, although even if the constants agree it would not necessarily follow that the enzyme is in the same form before and after the reaction or that a persistant contaminant is not present. A good indication as to the effect an enzyme may have on the reaction may be derived from following the catalysed reaction from both directions, that is, from the reactant side, as in the case of peptide bond formation, and from the product side, as in the case of dipeptide hydrolysis. A perfect catalyst would produce identical equilibrium constants from both directions. The effect of a contaminant or the interference of the enzyme on the establishment of equilibrium may be accentuated by catalysing the reaction with various concentrations of enzyme. However, proof of perfect catalysis only exists when it can be

demonstrated that the equilibrium attained is the same whether an enzyme interferes or not, or when the equilibrium constant calculated from independent data obtained without the use of enzymes agrees with that observed in the enzyme reaction.

References to the effect of enzymes on the equilibrium of a reaction are few, but in all cases quoted, the enzymes were found not to affect the equilibrium. Borsook and Schott (48) demonstrated that succinate dehydrogenase acted as a perfect catalyst when calculated free energy changes derived from a reaction catalysed with enzymes from four different sources all closely agreed. The same authors also showed that fumarase (49) and aspartase (50)had no effect on the equilibrium by a similar comparison of figures derived from reactions catalysed with enzymes isolated from various sources. Green and Stickland (51) showed that the same equilibrium point between molecular and ionic hydrogen was reached in the presence of an E. Coli dehydrogenase and a platinum black catalyst. Studies by Frantz and Loftfield with a dipeptidase and a carboxypeptidase were also reported as not to affect the position of equilibrium (52). Thus, to date, the few enzymes tested seem to act as perfect catalysts.

(b) The Reversibility of Enzyme Catalysed Peptide Hydrolysis

The enzymic synthesis of peptide bonds may be accomplished in two ways. The peptide bond may be synthesized by a specific ligase enzyme which combines two amino acid molecules to produce a third dipeptide molecule with the concurrent hydrolysis of ATP.

ATP is used to increase the rate of reaction and shift the equilibrium in favour of dipeptide production by activating the carboxyl component of the amino acid and producing a high energy species which is susceptible to nucleophilic substitution. However, since ATP is used to drive the reaction in the direction of synthesis, a true thermodynamic equilibrium between amino acids and dipeptide never exists, it being an equilibrium between amino acids, dipeptide, ATP, ADP and phosphate.

In order to measure the equilibrium constant of a dipeptide/amino acid system a peptidase must be employed to catalyse the reaction. Peptidases are classed as those enzymes which hydrolyse peptide bonds. However, as enzymes are reported to act as perfect catalysts, peptidases should be freely reversible and synthesise as well as degrade peptide bonds.

The synthetic activity of proteolytic enzymes has been extensively investigated. The first reported incident of peptide formation using proteolytic enzymes was probably the work of Danilewski in 1886 (53) who described the enzymic synthesis of a protein-like material, later called plastein. Plastein is an insoluble high molecular weight polypeptide formed by the condensation of small peptides when pepsin or papain is added to a concentrated partial hydrolysate of protein. Positive evidence of peptide bond synthesis in reactions catalysed by proteolytic enzymes was demonstrated by Wasteneys and Borsook (54). They found the synthetic action of pepsin and trypsin to have an optimum at pH 4, and be favoured by

a rise in the reaction temperature and substrate concentration. Further evidence was supplied by Bergmann and his group (46) (56) (57), who, in a series of papers not only demonstrated the synthetic abilities of the intracellular proteinases papain, cathepsin and bromelin, but also the synthesizing properties of the tryptic enzyme chymotrypsin, previously supposed to perform exclusively hydrolytic reactions under physio-chemical conditions. In fact it was noted (56)(57) that the conditions of pH, concentration, temperature and activation, which permitted synthesis by papain and chymotrypsin, were identical to those usually employed in proteolytic experiments suggesting that during the scission of peptide linkages the recombination of amino acid residues was simultaneously occurring.

Two types of synthesis were found to be catalysed by proteolytic enzymes. There was a synthetic reaction which was the reversal of peptide bond hyrolysis and a transamidation reaction. These two processes may be best illustrated by reference to the reaction between benzoyl-L-leucine and L-leucine anilide or glycine anilide with activated papain (56). In the reverse hydrolysis reaction benzoyl-L-leucine and L-leucine anilide react to form benzoyl-L-leucyl-L-leucine anilide. In the transamidation reaction the product of the reaction of benzoyl-L-leucine and glycine anilide is benzoyl-L-leucine anilide, having been formed by the replacement of the glycine residue of the original anilide by benzoyl-L-leucine. These reactions illustrate the specificity of the protease. A similar reaction was demonstrated with chymotrypsin by Fruton, Johnson and Fried (3). Benzoyl-L-tyrosine and glycinamide reacted

to form the condensation product benzoyl-L-tyrosylglycinamide in the presence of chymotrypsin. When benzoyl-L-tyrosinamide was substituted for benzoyl-L-tyrosine, the product was again benzoyl-L-tyrosylglycinamide, the chymotrypsin having catalysed the transamidation reaction leading to the replacement of the amide NH_2 group of benzoyl-L-tyrosinamide by glycinamide. The enzyme acted as an endopeptidase when it formed benzoyl-L-tyrosylglycinamide from benzoyl-L-tyrosine, since the product contained an internal peptide bond isolated from terminal amino or carboxyl groups and as an exopeptidase, in particular an aminopeptidase, when it hydrolysed the amide bond on benzoyl-L-tyrosinamide before forming the product. Usually proteolytic enzymes are specific in their peptidase activity, exopeptidases, such as leucine aminopeptidase or carboxypeptidase, requiring the presence of terminal amino or carboxyl groups for action, and endopeptidases, such as pepsin, requiring adjacent peptide bonds for action, i.e. isolation from terminal amino or carboxyl groups.

The properties of an enzyme required to catalyse a proteolysis at elevated temperature are somewhat specialized. Enzymes are usually obtained from living organisms operating at physiological temperatures which vary from about $10^{\circ}C$ up to about $40^{\circ}C$. An investigation of chemical reactions at temperatures up to $80^{\circ}C$ requires the use of enzymes adapted to high temperatures, usually those isolated from thermophilic organisms.

2:2 THERMOPHILIC ENZYMES

(a) Thermophilic Organisms

The highest temperatures on the surface of the earth, other than those of volcanic origin, occur in the hot spring areas, such as Yellowstone National Park in North America, the hot springs of Atagawa, Japan, and in New Zealand and Iceland. There have been numerous reports of algae and bacteria in these springs, many claiming that micro-organisms grow at temperatures as high as 89°C. Work by Kempner (59) in 1963 at the hot springs in Yellowstone National Park has, however, indicated that the highest temperature at which thermophilic algae can thrive is 73°C. All hot springs and run-offs above this temperature were perfectly clear of growing algae. In a series of experiments utilizing the fact that ^{32}P is incorporated into actively metabolizing algae he demonstrated that there was no active growth above 73°C. Similar work by Brock (60) has confirmed these results and extended them to show that the thermophilic algae have evolved so that their optimum metabolic temperature is the same as their environmental temperature, and so algae growing at the maximum temperature of 73°C metabolized optimally at that temperature. A precise explanation for this temperature maximum is unknown but it has been interpreted as either being due to the limitation of amino acid acceptance by soluble RNA and not specifically caused by protein or nucleic acid denaturation (59) or, since non-photosynthetic bacteria have been found at higher temperatures, that some aspect of the photosynthetic apparatus is inhibited above 73 - 75°C (60). Whatever its molecular basis, it is clear there is a maximum

temperature for active life processes and reports of organisms growing at temperatures higher than 75°C have been interpreted as survival without metabolism (59).

Thermobiosis at temperatures up to 50°C is found among algae, arthropods, fish, fungi, molluscs, nematodes and insects, as well as bacteria. Few, if any, plants are truly thermophilic, although there are examples such as the blue-green algae (61)(60).

There are few generalizations about thermophilic organisms apart from the fact that they are usually small in size. It has been demonstrated by Lamanna (62) that, for the Bacillus genus, size decreases as the temperature range for growth rises. Copeland (61) has shown this also to be true for blue-green algae where thermophilic cells tend to be smaller and more slender than mesophilic cells.

Thermophilic aerobic spore forming bacteria are the most common and accessible organisms capable of growth at elevated temperatures (63). The temperature conditions for bacterial growth are obviously most favourable in hot springs, but the supply of organic matter is low and, as a result, autotrophic thermophilic bacteria are rare in such places unless masses of algal mat accumulate and decay. In fact thermophilic bacteria can be found in almost every sample of soil, mud or water examined (63) and particularly occur in masses of plant material such as haystacks or manure piles containing considerable straw. The most

commonly found genus of thermophilic bacterium is Bacillus.

(b) Thermophilic Enzymes

Studies by Militzer and his group (64)(65)(66)(67) have clearly established that some, but not all, of the enzymes from thermophiles are more heat resistant than enzymes from mesophilic sources. They have demonstrated the presence of thermostable respiratory enzymes in Bacillus stearothermophilus, most of the enzymes being found together in a granule (66) within the cell, although a soluble apyrase was prepared (65) indicating that combination within the particle was not necessary for thermostability. The only freely soluble thermostable enzymes found were hydrolytic in nature.

A variety of thermophilic enzymes have been isolated from various sources, a selection of which have been presented in Table 2:1.

The stability of thermophiles and thermophilic enzymes to elevated temperatures is due to a combination of factors. Allen (80) has suggested that although many of the proteins of a thermophile might be readily denatured at temperatures where the cell grows well, the cell maintains its viability by rapid re-synthesis or repair of the inactivated protein. There can be no doubt that thermophiles are more stable in a growing condition than after growth ceases, but the simple increase in the temperature

Table 2:1 THERMOPHILIC ENZYMES AND THEIR SOURCES

Organism		Properties		Ref
Blue-green algae				
eg. Cyanidium caldarium	Yellowstone Park USA	Optimally adapted to temperatures up to 73°C		60
Fungi				
eg. Talaromyces duponti	Aminopeptidase	Opt. temp 55°C	Activated by Co^{2+}	
	m.wt. 400,000	Opt. pH 6.9	Inactivated by EDTA	68
Bacteria				
Actinomyces		Opt. temp 60-80°C	Activated by Ca^{2+}	
Micromonospora vulgaris	Crude enzyme extract	Opt. pH 4.7 or 8.5	Inactivated by heavy metals and PCMB	69
Thermomonospora fusca	Purified 30-40 fold Proteolytic activity	Opt. temp 60-70°C	Inibited by PCMB	70
	m. wt. 21,500-23,800	Opt. pH 8.5-9.0		
Thermus aquaticus	Crude preparation Aspartokinase	Opt. temp 70°C	Maintained allosteric inhibition at high temps	71
Bacillus				
B. stearothermophilus	Amylase: In vivo study	Opt. temp 50-60°C		
		Opt. pH 7.3-7.5		72

Table 2:1 contd

B. stearothermophilus	Purified 20-40 fold Proteolytic activity	Opt. temp 55°C Opt. pH 6.9-7.2	Activated by Ca^{2+} or Mn^{2+} Inhibited by PCMB	73
" " "	Aminopeptidase (API) m. wt. 360,000-400,000	Opt. temp. 80-90°C Opt. pH 7.5-9.5	Broad specificity Activated by Co^{2+} Inhibited by EDTA	74 75 76
B. thermoproteolyticus	Thermolysin	Opt. temp. 60°C	Activated by Ca^{2+}	77 78
" " "	Protease m.wt. 37,500	Opt. pH 7.0	Limited specificity	79

characteristic of growth cannot totally account for thermophily (81).

The inherent stability of the proteins of thermophiles has been amply demonstrated in recent years by a number of workers. Campbell (82) isolated crystalline amylase from cultures of Bacillus coagulans grown at 55°C and found it was markedly more heat stable than a similar isolate from cultures grown at 35°C. Koffler (83) compared flagella from thermophilic and mesophilic bacteria and found that thermophilic flagella were considerably more stable to temperature and to denaturation agents, such as urea and acetamide. This suggested that the relative stability of the flagella was due to more numerous, stronger, or more strategically located hydrogen and hydrophobic bonds. Allen (84) recorded the fact that the decrease in growth factor requirements of some thermophilic bacteria in media containing relatively high concentrations of calcium or magnesium ions was suggestive of a protective effect by these divalent ions. Indeed the importance of divalent ions to thermophilic enzymes is emphasized by the data presented in Table 2:1. The majority of the isolated thermophilic enzymes require either calcium, cobalt or manganese ions for activity and are inhibited by metal chelating agents such as ethylenediaminetetra acetic acid (EDTA) and p-chloromercuribenzoate (PCMB). The role of divalent ions is thus associated with the stability of the enzyme molecule at elevated temperatures.

The choice of enzyme as catalyst for these equilibrium studies was governed by two factors. Firstly the choice of substrate and

the specificity of the enzyme for that substrate, and secondly the ease of preparation or availability of that enzyme.

There are two types of substrate which can be utilized in this study depending upon the precise reaction to be studied. Unprotected amino acids and peptides can be employed. The advantages of these compounds are the ease of obtaining radio-labelled reactants, necessary for the accurate determination of equilibrium constants, and the relatively high solubility of the peptides. The disadvantages, however, are that depending upon the amino acids chosen, the reaction products formed would certainly be a mixture of polymers of varying length and composition and could possibly include branched chain or closed chain peptides. This would make the isolation of the reaction mixture components very difficult and an accurate thermodynamic description of the reaction almost impossible. Difficulty would also be encountered from the different states of ionization of the reactants and products, more energy being required to react two ionized amino acids than two peptides, or an amino acid and peptide, where the ionized groups are further apart. The resultant free energy of peptide bond formation from such a system would be the average value of a variety of different bond energies, the value varying as the length and complexity of the peptide product varied. Using partially protected amino acids as reactants bestows several advantages upon the reaction system used for equilibrium measurements. By protecting the carboxyl group of one amino acid and the amino group of the second amino acid, the only possible peptide bond forming reaction between

them, provided they are not acidic or basic amino acids, is that between the two unprotected groups to form a fully protected dipeptide. This reaction may be accurately defined thermodynamically. It also requires less energy to form an unionized dipeptide from the two partially ionized amino acids, shifting the equilibrium slightly to the right, favouring dipeptide formation and making measurements easier (2). The character of the peptide bond formed between two partially blocked amino acids is constant within that reaction system and has been taken to correspond to the interior peptide linkages of proteins (85). The disadvantages of this system arise from the fact that the solubility of the dipeptide, and to some extent the amino acids, is markely decreased by the inclusion of protecting groups, and as a result, equilibrium measurements have to be made in dilute solution. The radiolabelling of protected amino acids and dipeptides is also more difficult since they have to be synthesized in the laboratory from precursors. However, the advantages of the protected amino acids and dipeptides make them by far the better reactants for thermodynamic equilibrium studies.

The choice of enzyme is governed by its availability and its specificity. Thermophilic bacteria are the primary source of thermophilic enzymes (See Table 2:1), since they are the commonest accessible organisms which grow at elevated temperatures (84). The most widely distributed thermophilic bacterial species are the bacilli, the majority of work being concentrated on two particular strains, Bacillus stearothermophilus and Bacillus thermoproteolyticus.

It was decided to investigate the use of enzyme catalysts isolated from these two bacteria for the equilibrium studies.

2:3 THE ISOLATION AND CHARACTERIZATION OF THERMOPHILIC ENZYMES

(a) Bacillus stearothermophilus

B. stearothermophilus has been reported to yield several different thermophilic enzymes. Hartman (72) selected it for study as it gave higher yields of amylase activity than 168 other strains of stenothermophilic bacteria tested. The first major attempt to purify and study the properties of proteolytic enzymes isolated from this bacterium was carried out by O'Brien and Campbell (73). The bacterium was cultured in peptone/yeast extract medium and the enzyme preparation isolated from the culture supernatant liquid by ammonium sulphate fractionation, calcium phosphate gel fractionation and precipitation in ethanol. Casein hydrolysis was used to determine optimum operating conditions for the enzyme extract, 55^{o}C being the optimum temperature and the optimum pH being 6.9 to 7.2 An absolute requirement for calcium or manganese ions for activitiy was demonstrated. The enzyme extract hydrolized, in addition to casein; gelatin, alpha soy protein, alkaline haemoglobin, and the synthetic substrates L-leucylglycylglycine, triglycine, tetraglycine and glutathione. This broad specificity was taken to indicate that at least two types of enzymes were present, an endopeptidase and an exopeptidase. B. stearothermophilus was also the source of the purified protease isolated by Roncari and Zuber (74). They purified a membrane bound thermostable aminopeptidase

by fractionation on Sephadex G 150, heat treatment, chromatography on DEAE Sephadex, fractionation on Sephadex G 200 and preparative polyacrylamide gel electrophoresis. The purified enzyme operated over a broad temperature range, from 20°C to 90°C, had a pH optimum between 7.5 and 8 for leucine *p*-nitroanilide hydrolysis and between 9.2 and 9.4 for glycyl-L-leucyltyrosine hydrolysis, and exhibited an absolute requirement for cobalt ions for activity.

It was decided to culture B. stearothermophilus, prepare a crude enzyme extract from it, and study the specificity of the extract with the intention of isolating a purified enzyme for use in the equilibrium studies.

Bacillus stearothermophilus (strain NCIB 8924) was supplied by the National Collection of Industrial Bacteria, Torrey Research Station, Aberdeen, as a freeze-dried culture and was initially grown on nutrient agar plates and slopes at 55°C. The resultant cells were inoculated into Brain Heart Infusion medium (BHI) (Oxoid Ltd., London) (76) but it was found that this medium had a poor buffering capacity and that the pH of the medium was a sensitive function of temperature with the result that the pH dropped from 7.2 at 25°C to 6.0 at 55°C and no growth occurred, probably because of the limited pH tolerance of B. stearothermophilus (72). Neutralization of the BHI medium at 55°C with 0.1 M sodium hydrogen orthophosphate did not produce any improvement in growth. In order to test whether sporulation was responsible for the poor growth, spore breaking techniques and a spore stain (86) were tried, both with negative results. The BHI medium was discarded and Lab

Lemco medium (L.L.)(Oxoid Ltd., London) tried (74). This produced successful growth of the Bacillus, the maximum yield of cells being obtained after 18 hours culture. The technique was scaled up to provide sufficient bacteria for enzyme separation. Cells were inoculated from nutrient agar slopes into 4 x 250 ml L.L. medium and incubated at 55°C with shaking. After 18 hrs the cultures were combined and made up to 10 litres in a fermentation vessel. This was aerated at a rate of 3 litres per minute using a vibro mixer (Shandon Scientific) and the vessel kept at 55°C in a thermostatically controlled water bath. After a further 18 hours the cells were harvested using a Sharples centrifuge to give a yield of approximately 0.75 g bacterial cells (wet weight) per litre of medium. The cells were washed with 20 ml distilled water, recentrifuged at 7,500 x g for 8 minutes and stored in 1.0 g batches at -15°C. In order to release any membrane bound proteases the cells were treated by sonication. 1.0 g of bacterial cells were suspended in 3.0 ml of 0.05M Tris-HCl buffer, pH 7.2, 0.001M with respect to cobalt chloride, and were given six bursts of 20 sec each from a sonic vibrator, the temperature of the mixture being kept below 10°C. The vibrator was one constructed at the School of Physics, University of Bath, and consisted of a 200 watt amplifier driving a 20 Kc oscillator and harnessed to a magneto-strictive transducer unit with a titanium stud attached. The cellular debris was centrifuged off at 12,000 x g for 10 minutes and the supernatant stored at 4°C. The enzyme extract retained its activity in this state for up to two weeks.

The activity and specificity of the enzyme extract was measured by its ability to hydrolyse a selection of dipeptide substrates. The efficiency of hydrolysis was assessed chromatographically. The following substrates were used to assess the specificity of the extract: L-leucylglycine; L-leucyl-L-tyrosine; glycyl-L-phenylalanine; L-alanyl-L-alanine; CBZ-L-leucylglycyl methyl ester; CBZ-L-leucyl-L-tyrosyl amide; (Sigma London Chemical Co. Ltd.,) and N-acetylglycyl-L-phenylalanine methyl ester (Cyclo Chemicals; U.K. distributors, Cambrian Chemicals Ltd.,) The assay system consisted of 5.0 ml 0.03M dipeptide substrate (or as high a concentration as possible for the insoluble substrates, CBZ-L-leucylglycine methyl ester, CBZ-L-leucyl-L-tyrosine amide and N-acetylglycyl-L-phenylalanine methyl ester) in 0.05M Tris-HCl buffer, pH 7.2 with 0.001M cobalt chloride, and 1.0 ml enzyme extract. The solution was incubated at 65^{o}C and 1.0 ml samples removed at five minute intervals and stored on ice. 10 µl of each sample was then applied to Whatman No. 1 paper along with appropriate standards and the chromatogram was developed in butanol/acetic acid/water-40/10/5 v/v/v. After drying, the spots on the chromatogram were visualized with either 0.1% ninhydrin in methyl cellusolve, to detect free amino groups, or chlorine-tolidene spray (87) for carbobenzoxy compounds.

The enzyme extract was found to completely hydrolyse the unprotected dipeptides but none of the protected dipeptides. It was concluded that the majority of the enzyme activity in the crude

extract was of exopeptidase character and so of no particular value for these studies.

(b) Bacillus thermoproteolyticus - Thermolysin

The proteolytic enzyme of prime importance isolated from Bacillus thermoproteolyticus is known as Thermolysin. Thermolysin was first extracted and purified by Endo (88). Recent studies (89)(90)(91) have shown it to be a polypeptide chain 316 residues long of molecular weight 34,600. It has a complete absence of cystine or cysteine and only two methionyl residues, but there is no unique feature of its sequence that can be related to its thermal stability. The active site of the molecule contains a zinc atom, and four calcium atoms are necessary for stabilization. The removal of the calcium atoms causes loss of activity and decrease in helical content (78) and Feder, Garrett and Wildi (92) have demonstrated that these ions are required for thermal stability, playing an important role in maitaining the integrity of the tertiary structure at elevated temperature. The enzyme operates over a temperature range of 25 - 90^{o}C (77) but at temperatures above 80^{o}C affinity for the substrate falls probably due to conformational changes caused by thermal denaturation. Thermolysin exhibits a tolerance to pH over the range pH 5 - 10 (78), the optimum activity occurring between pH 6.5 and pH 8.5 The substrate specificity of thermolysin is limited. It preferentially hydrolyses peptide bonds involving the amino groups of hydrophobic amino acid residues with bulky side chains (94). Susceptibility

to hydrolysis also requires the absence of free amino and carboxyl groups from the immediate vicinity of the peptide bond to be hydrolysed. Ambler and Meadway (95) demonstrated that besides hydrolysing peptide bonds on the N terminal side of valine, leucine, isoleucine, and phenylalanine residues, N terminal methionine tyrosine and some alanine bonds are split. A small proportion of N terminal asparagine, threonine, histidine and glycine bonds are also susceptible. Thermolysin also requires the L configuration at the sensitive residue and ω amino and and caroboxyl groups only alter the activity slightly (94). Thermolysin does not have amidase or esterase activity and it does not cleave peptide bonds at the amino site of a hydrophobic residue which has proline in the carboxyl position (94). The specificity is the same at extremely different temperatures. The specificity to synthetic substrates was examined by Matsubara (79) and by Morihara and Tsuzuki (96) who found that sites of action of the enzyme were the same as those of natural substrates.

During the course of this study a commercial source of thermolysin became available enabling its suitability for equilibrium studies to be conveniently determined. Thermolysin specificity and activity were assessed by dipeptide hydrolysis both chromatographically and colo rimetrically.

The substrates tested with thermolysin were: L-leucylglycine; CBZ-L-leucylglycine methyl ester; CBZ-L-leucyl-L-tyrosine amide ; CBZ-glycyl-L-phenylalanine amide; (Sigma London Chemical Co. Ltd);

N-acetylglycyl-L-phenylalanine methyl ester; CBZ-L-threonyl-L-leucine amide (Cyclo chemical; U.K. districutors, Cambrian Chemicals Ltd.) and casein (BDH Ltd.) Thermolysin was obtained from Calbiochem Ltd., as a three times crystallized product containing 50.2% enzyme protein, 21.3% acetate salts and a trace of water. It was used without further purification. All other chemicals used were of Analar grade.

The reaction system employed was similar to that of Matsubara (79). The synthetic substrates (5×10^{-3}M) or casein (2%) were dissolved in 2.0 ml 0.01M Tris-HCl buffer, pH 8.0 with 0.01M calcium chloride, 0.2 ml ethanol being added to aid the solubility of the CBZ dipeptides. The thermolysin was prepared as per Matsubara (79) to a concentration of 0.2 mg/ml. 2.0 ml substrate were incubated with 0.1 ml enzyme solution, at 60°C in the case of the synthetic substrates and at 40°C in the case of casein. Aliquots were removed at timed intervals and stored on ice. The samples were assayed using chromatography and a colorimetric ninhydrin method.

Chromatography: 10 µl samples were applied to either Whatman No. 1 paper or a thin layer chromatography plate coated to a thickness of 0.2 mm with Silica G.F. (Merck: U.K. distributors, Andermann & Co), along with appropriate standards. The chromatograms were developed in butanol/acetic acid/water, 48/12/20, v/v/v, dried and visualized with either 0.1% ninhydrin in acetone or chlorine-tolidine spray (87). The results indicated

that, of the substrates tried, thermolysin would only hydrolyse casein, CBZ-L-threonyl-L-leucine amide and CBZ glycyl-L-phenylalanine amide.

Ninhydrin method: Based on the colourimetric method of Cocking and Yemm (97) this technique gave an indication of the rate of hydrolysis of the susceptible substrates. The reagents used were 5% w/v ninhydrin in 2-methoxyethanol; 0.2M sodium citrate buffer, pH 5.0 and 0.01M aqueous potassium cyanide,diluted 1:50 with 2 methoxyethanol before use. The assay system consisted of 0.1 ml reaction sample, 0.9 ml distilled water, 0.5 ml citrate buffer, 1.0 ml dilute cyanide and 0.2 ml ninhydrin solution. The solutions were heated in a boiling water bath for 25 minutes, cooled and the optical densities read at 570 nm. Of the two synthetic substrates hydrolysed by thermolysin, the amino acid product CBZ threonine, from CBZ-L-threonyl-L-leucine amide, was found to interfere with colour development in the ninhydrin assay and so no reaction rates could be determined for that hydrolysis. However, after preparing a ninhydrin calibration curve of phenylalanine amide, the rate of hydrolysis of CBZ glycyl-L-phenylalanine amide by thermolysin at a final enzyme concentration of 10 µg/ml (approx. 1×10^{-7}M) was determined as 3.5 µm/min/mg enzyme at 60^{o}C.

It was decided that the system to be employed for the equilibrium studies would be the formation and hyrolysis of protected dipeptides catalysed by the endopeptidase, thermolysin.

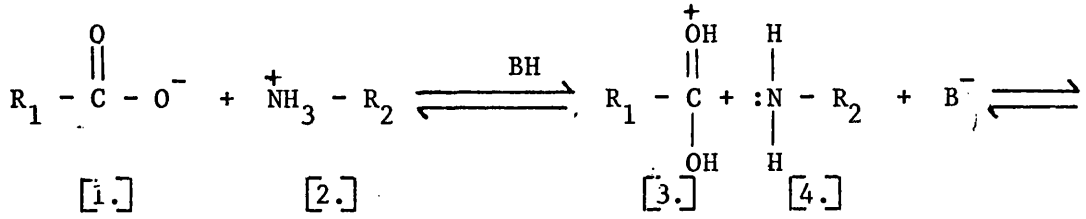
CHAPTER 3

THE SYNTHESIS OF PROTECTED AMINO ACIDS AND DIPEPTIDES

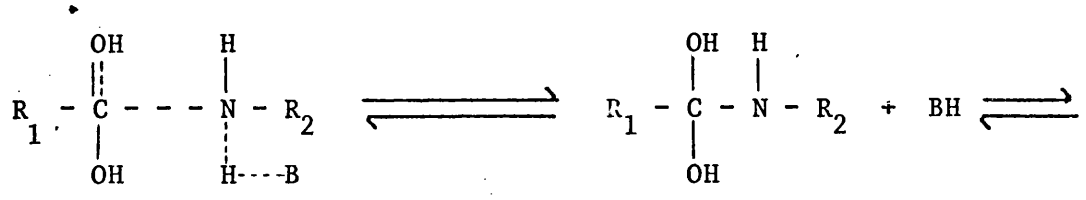
3:1 INTRODUCTION

The reaction under consideration in this work, peptide synthesis by the reversal of hydrolysis, is characterized by the formation of a peptide bond between the carboxyl group of one amino acid and the amino group of a second amino acid: The thermodynamically unfavourable aspect of this reaction may be illustrated by considering it in mechanistic terms: Equation 3:1 (adapted from (98)).

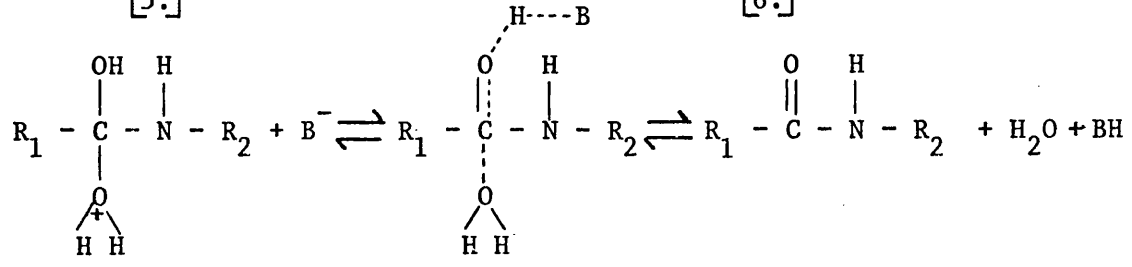
Equation 3:1



[1.] [2.] [3.] [4.]



[5.] [6.]



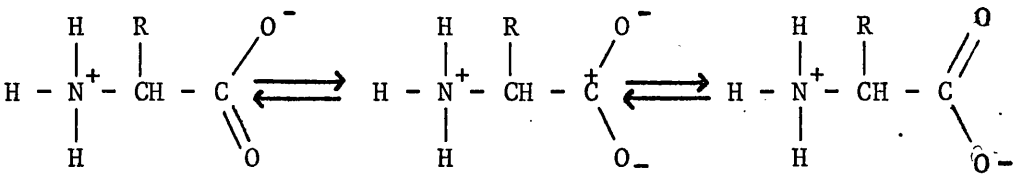
[7.] [8.] [9.]

The gain of a proton by the negatively charged oxygen atom of the carboxyl group of one amino acid [1] and the loss of a proton by the amino group of a second amino acid [2] and the weak acid BH, intitiates the reaction. These proton transfers probably do not occur directly between the molecules but via adjacent solvent water molecules. This reaction is very slow, the equilibrium favouring the ionization of the two amino acids. The formation of a positive charge on the carboxyl group of the first amino acid [3] promotes nucleophilic attack via the free electron pair on the second amino acid [4] to form a transition state [5] which is rapidly reversible between the dimer [6] and the monomers [3] and [4] The formation of the tetrahedral intermediate [6] , so named because the four groups around the carbon atom are almost tetrahedrally disposed about it, may lead to protonation of one of the hydroxyl groups [7] , by the weak acid, a reaction whose equilibrium lies in favour of the unionized intermediate [6] . The protonated form of the tetrahedral intermediate [7] then undergoes an elimination reaction via the unstable intermediate [8] to form the dipeptide [9] and water. The rate determining step of this reaction is the initial proton transfer, inducing activation of the carboxyl group by charge suppression. This step is energetically unfavourable.

Peptide synthesis with free amino acids according to equation 3:1 cannot proceed unambiguously. The activated amino acid would react not only with the functional group of the second amino acid, but also with the amino groupson its own molecules forming a variety of homo- and hetero- peptides. To prevent this uncontrolled polycondensation the amino and carboxyl groups not taking part in the reaction must be protected or blocked. Similarly all other functional groups which might interfere with peptide bond formation should be masked. The residues used for blocking are called amino- or N-protecting groups and carboxyl- or C- protecting groups.

In addition to protecting the amino and carboxyl groups, the blocking residues suppress zwitterion formation (Equation 3:2) in the amino acid.

Equation 3:2

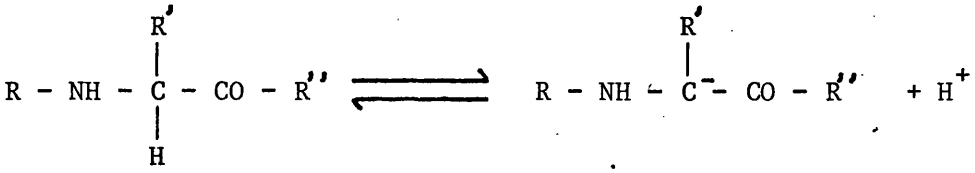


Activation of the carboxyl group in an unprotected dipolar molecule (Eq. 3:2) is difficult and the addition of the amino nitrogen similarly problematical. The blocking of one of the functional groups of the amino acid deprives it of the ability to form an inner salt, the other functional group then becoming

readily available for reaction.

Blocking groups are also used to prevent racemization during peptide synthesis. All amino acids, with the exception of glycine, have at least one asymmetric carbon atom which is susceptible to optical transformation, this being the result of a loss of a proton from the α-carbon atom: Equation 3:3 (99).

Equation 3:3



Generally the only susceptible asymmetric centre is the α-carbon adjacent to the activated carboxyl group. The stability of the C - H bond is strongly influenced by the substituent attached to the amino group, thus protecting groups have to be chosen carefully to suppress racemization.

The protecting groups attached to the amino acids and dipeptides used in this investigation must fulfil several functions:

(i) the protecting groups must be compatible with the enzyme, thermolysin.

(ii) they must be easily attached to the amino acids, since the radiolabelled amino acid and dipeptide substrates required for this study must be synthesised from labelled precursors.

(iii) the blocking groups must retain their protecting properties under a variety of conditions, i.e. prevent racemization during enzymic peptide synthesis at elevated temperatures and during the chemical synthesis of the radiolabelled substrates.

There is a wide choice of protecting groups, the more important ones, and their properties, are described below.

3:2 PROTECTING GROUPS IN PEPTIDE SYNTHESIS

(a) Amino protection. An amino protecting group prevents reaction by reducing the normal nucleophilicity of the amine by decreasing the availability of its unshared electron pair.

1. *The Carbobenzoxy group (CBZ group).* The carbobenzoxy or benzyloxycarbonyl group is by far the most important blocking group in peptide chemistry. It is easily introduced into the amino acid, readily accessible for further reaction, is practically universally applicable in peptide synthesis, and it has numerous convenient ways for its removal.

Carbobenzoxy chloride reacts in aqueous alkaline solution with amines or amino acids to form the corresponding urethanes in high yield. There are only a few cases where this reaction does not proceed satisfactorily. The CBZ group may be used to block not only the α amino group, but simultaneously the α and ε groups of lysine or the α and δ groups of ornithine. Carbobenzoxy-amino acid derivatives are important starting materials for peptide synthesis. For instance, the esters may be prepared either by the esterification of the blocked amino acid or by the carbobenzoxylation of amino acid esters. CBZ amino acids may be used for peptide synthesis with practically no limitations and the CBZ group has the special advantage of providing protection against racemization. CBZ groups are sensitive to strong alkali, especially in peptide esters containing glycine when hydantoins are formed. However it is possible to remove selectively a carboxyl ester group by alkaline hydrolysis under controlled conditions and leave the CBZ moiety intact. The CBZ group is removed by catalytic hydrogenation under a variety of conditions. It is also cleaved by strong acid conditions, with the resultant esterification of any free carboxyl groups present. Peptide bond stability during decarbobenzoxylation is governed by the solvent and conditions under which the process is carried out.

2. *The tert-Butoxycarbonyl Group (t-BOC group).* The second most successful N-protecting group is the tertiary butoxycarbonyl residue, an aliphatic urethane compound. The

t-BOC group cannot be introduced into the amino acid via its chloride as it is too unstable. t-BOC amino acids are most often synthesized by aminolysis of a suitable t-butyloxy-carbonyl aryl ester, usually p-nitrophenyl ester. Preparation from t-BOC azides and imidazoles is occasionally employed and the use of t-butyl pentachlorophenol carbonate has been recently introduced. t-BOC amino acids cannot be esterified by alcohol in the presence of an acid since the protecting group is very acid labile. Cleavage by acid is complete within 30 minutes at room temperature. The t-BOC group is stable to catalytic hydrogenation and strong alkali.

A similar group to the t-BOC residue is the tertiary amyl oxycarbonyl (t-AOC) protecting group. This has been suggested as a more convenient blocking group than t-BOC in many cases as it may readily be introduced into amino acid esters using the tert-amyl chloroformate. It is easily removed by acid cleavage.

3. *The p-Toluenesulphonyl Group (Tosyl group).* The p-toluenesulphonyl or tosyl group was probably the first successful application of removable protection for amino groups. Tosyl amino acids are prepared in alkaline solution from toluene sulphonyl chloride and the corresponding amino acid. It is stable to catalytic hydrogenation and alcoholic HCl but is removed by reduction with sodium in liquid ammonia. The tosyl group has several drawbacks. In the synthesis of peptides it

cannot be coupled to the amino component of a peptide by the generally used mixed anhydride procedure. In general, tosyl amino acids are reacted as acid chlorides, but under mildly alkaline aqueous coupling procedures decomposition of the tosyl group may result. Tosyl amides are also prone to accept second acyl groups on their nitrogen atom.

4. *Other N-Protecting Groups.*

i) The *formyl* group is introduced into an amino acid by reaction in a mixture of formic acid and acetic anhydride. This group is resistant to a variety of reagents including hydrobromic acid in acetic acid, sodium in liquid ammonia and mild alkali. Racemization problems and instability of the formyl group under certain coupling conditions restrict its use.

ii) The *trifluoroacetyl* (TFA) group is important since it is the only major amino blocking group which can readily be removed by alkali. The TFA amino acid is prepared by reacting the amino acid in trifluoroacetic anhydride at -10° to $10^{\circ}C$ with trifluoroacetic acid as solvent. Amino acids may also be trifluoroacetylated with ethyl thiotrifluoroacetate. The TFA group has only limited application possibly because of difficulty in peptide bond formation with the protected amino acid.

iii) *Phthalyl* protected amino acids are synthesized by fusion of the amino acid with phthalic anhydride at temperatures around $150^{\circ}C$. Phthalylation may also be achieved by reacting amino

acids with N-ethoxycarbonyl-phthalimide, it being less susceptible to racemization than the anhydride process. The phthalyl amino group is resistant to acids but unstable to alkali.

iv) The *triphenylmethyl* or *trityl* group is not an ideal amino protecting group but is important because of its ready cleavage property, enabling it to be selectively removed even in the presence of t-BOC groups. Trityl chloride is used as the alkylating agent, tritylation occurring most readily if amino acid esters are used. Trityl amino acids may also be obtained by the catalytic hydrogenation of benzyl esters, but the reaction must be stopped before hydrogenolysis of the trityl group occurs. The influence of the large trityl group makes peptide synthesis with trityl amino acids difficult, due to steric hinderance, but is successful with di- and poly- peptides. The trityl group is removed under mildly acidic conditions.

(b) Carboxyl protection. The blocking of the carboxyl group in an amino acid is not primarily for protection, but to release the amino acid from its zwitterionic form. Peptide coupling may be carried out without carboxy protection provided the activating and coupling steps are separate.

1. *Esterification.*

(a) *Methyl and Ethyl Esters.* There is little difference in preparation and application between methyl and ethyl esters. Esterification is obtained by reacting the amino acid in excess alcohol in the presence of an acid catalyst, such as

hydrochloric acid or thionyl chloride. A methyl or ethyl ester hydrochloride results. Esterification under more vigorous conditions leads to dimethyl and diethyl ester formation. N-blocked amino acid esters are usually produced by attaching the N-blocking group to the ester, the reverse procedure possibly resulting in lability of the protecting group by the acid catalysed esterification. Free amino acid esters are obtained from their hydrochlorides by reaction in aqueous alkali. However, such compounds are normally unstable, aminolysis leading to the formation of diketopiperazines. Higher peptide esters are relatively more stable. Ester linkages are susceptible to mild alkaline hydrolysis, peptide bonds usually being stable to this process. Hydrolysis becomes increasingly difficult with chain length with the resultant risk of instability of N-protecting groups and racemization. Hydrolysis by acids and enzymes also occurs.

b) *Tertiary Butyl Esters*. These esters are a relatively recently introduced carboxyl blocking group and have become important because of the ease of hydrolysis of the group with acid. The bulky tert. butyl group also confers stability from diketopiperazine formation on the protected amino acid due to steric hindrance. Tert. butyl amino acid esters may be synthesized by several methods. Esterification of the amino acid with isobutene catalysed by sulphuric acid is usually used. Transesterification of the free amino acid with tert. butyl acetate in perchloric acid is possible although better results are obtained with this method if N-protected amino acids are used.

Esterification may also be achieved with tert. butyl alcohol and phosphorus oxychloride in pyridine. The free tert. butyl ester of most amino acids are stable liquids considerably more difficult to hydrolyse in alkali than methyl or ethyl esters. Cleavage of the group occurs with hydrochloric acid in ethyl acetate.

c) *Benzyl Esters.* Benzyl esters of amino acids are usually obtained by reacting a mixture of amino acid and benzyl alcohol with p-toluenesulphonic acid as catalyst. Increased yield may be obtained by the removal of the water formed during esterification by azeotropic distillation. The benzyl ester may also be prepared through N-protected intermediates via the benzyl alcohol or by transesterification with benzyl borate and sulphuryl chloride as catalyst. The benzyl group is removed by catalytic hydrogenation.

2. *Hydrazide and Amide Formation*

(a) *Hydrazide formation.* The hydrazide group itself is not suitable for carbonyl protection as an amino group cannot be acylated selectively in its presence. The hydrazines used are blocked with amino protecting groups to prevent side reactions and the hydrazide formed can be employed in the chemical synthesis of peptide bonds using the azide coupling method. Typical hydrazide blocking groups are carbobenzoxyhydrazide and tert. butyloxy-carbonylhydrazide. The hydrazide cannot be selectively cleaved from the amino group.

b) *Amide formation*. The difference between an acid amide bond and a peptide bond is negligible. In general it is not possible to protect carboxyl groups reversibly by amide formation. Amides are synthesized by the ammonolysis of the amino acid or peptide ester. Alternative methods involving liquid ammonia or coupling methods are used in special cases. Cleavage occurs by acid hydrolysis under vigorous conditions, but other blocking groups and peptide bonds themselves are also affected.

3. *Salt formation*. Salt formation by the addition of a strong base to an amino acid solution is readily achieved and gives quantitative yields. The salt need not be isolated and the free acid for the next coupling step is easily obtained by acid treatment. Difficulty arises from the fact that amino acid salts are normally only soluble in water and in a coupling reaction hydrolysis of an activated amino acid salt would occur. Isolation of peptide products from a coupling reaction mixture is often difficult as both starting component and end product are present as acids.

The choice of protecting groups to be used in this study also depended upon the amino acid activation technique employed in the synthesis of the radiolabelled blocked dipeptides. The more important peptide bond forming methods are discussed below:

3:3 METHODS OF AMINO ACID ACTIVATION AND COUPLING

Chemical synthesis of a peptide bond is generally carried out by conversion of the carboxyl component of one amino acid by an activating agent into a more reactive acyl derivative which will react under mild conditions with the amino component of the second amino acid. Activation of the amino group for peptide synthesis is also possible but not widely used. Some coupling agents, such as dicyclohexylcarbodiimide, act by forming reactive carboxyl intermediates and then bringing about peptide bond formation simultaneously in the same reaction mixture.

1. *The Azide Method* of carboxyl activation was one of the first coupling methods developed. An acyl amino acid ester, usually the methyl or ethyl ester, is converted to the corresponding hydrazide by reaction with hydrazine, the product being purified by recrystallization. This is treated either with aqueous nitrous acid under acid conditions, or under anhydrous acidic conditions with an alkyl nitrite of nitrosyl chloride to form a reactive azide. Azides are very unstable and have to be handled at low temperatures and used as quickly as possible. The azide is reacted with an amino acid having a free amino group to give an acyldipeptide in average yield, approximately 50%. Side reactions can occur, such as the formation of urea derivatives from the Curtius rearrangement of the azide with the amino component of the amino acid, or amide formation. The azide method is unique in being the only coupling procedure where no racemization occurs.

2. *The Acid Chloride Method* is another of the early peptide synthesizing methods. The acylamino acid chloride is prepared by treatment with phosphorus penta- or tri-chloride or thionyl chloride. The activated derivative is then allowed to react with the amino component of a second amino acid, often at elevated temperature. Limitations of the method and undesired side reactions limit its use. The number of N-protected amino acids which may be used with this method are restricted. Gentle heating or long storage of the activated residue causes breakdown of N-protected compounds, (CBZ amino acid chlorides form Leuchs anhydrides), and extensive racemization. Tosyl acid chlorides (and azides) undergo fragmentation in dilute alkali. A general disadvantage arises from the contamination of the acid chloride with phosphorus oxychloride resulting in phosphorus containing peptide derivatives. As a result the acid chloride method is no longer extensively used.

3. *Mixed Anhydrides*, because of the tremendous variety of possible residues, are probably the best carboxyl activating groups available. Amino acyl mixed anhydrides are formed from acids with fairly long aliphatic chains or branched chains, for example, isovaleroyl or trimethylacetyl residues, with excellent yields and little by-product formation. They are usually prepared by addition of one mole of acid chloride to a cooled solution of one mole of N-protected amino acid and one mole of tertiary base, such as triethylamine, in non-polar solvent. Formation of the

anhydride is instantaneous, indicated by the precipitation of the basic salt, triethyl ammonium chloride. The mixed anhydride can be used in the acylating step without previous isolation or purification. The ease of preparation of the anhydride and the ready availability of acid chlorides is probably the reason for the popularity of the method. There is no limit to the number of possible mixed anhydrides since not only carboxylic acids, carbonic acid half esters, derivatives of phosphoric and phosphorus acids and sulphuric acid are used, but derivates of aliphatic and aromatic sulphonic acids are also available as partner acid. Mixed or asymmetrical anhydrides are of great practical value in the synthesis of smaller peptides especially since the second product of the acylation can be readily separated by crystallization of the main peptide product. However with longer peptides this separation becomes increasingly difficult, a consideration which led to the development of methods based on the aminolysis of esters.

4. *Active Esters.* Aminolysis of simple alkyl esters is usually slow. Phenol esters are more reactive because the phenolate ions, being less basic than alkoxide ions, are better leaving groups. Thus the acidity of a phenol, and therefore the reactivity of its esters towards nucleophiles such as amines, is significantly increased by the substitution of electron withdrawing groups into the aromatic ring. Both phenyl and thiol esters are used for carboxyl activation, the most extensively employed reagents being p-nitrophenyl esters. p-Nitrophenyl esters are

prepared by reacting N-protected amino acids with p-nitrophenol using dicyclohexylcarbodiimide as coupling agent. Unfortunately these reactions invariably suffer racemization. The N-protected amino acyl p-nitrophenyl esters produced are usually stable crystalline materials which, when mixed in organic solvent with the amino component of an amino acid at room temperature produce good yields of the corresponding peptide. Apart from conventional peptide syntheses, activated esters are frequently used in 'solid phase' synthesis.

5. *Carbodiimides* are termed coupling agents, as opposed to activating agents, since the addition of these compounds to a mixture of acid and amine results directly in amide bond formation. The reactive intermediates formed during these coupling reactions are almost certainly activated carboxyl derivatives. The method of peptide synthesis employing dialkylated carbodiimides was introduced by Sheehan and Hess (100). It has since become the most important and most widely used method of peptide synthesis largely because of the simple preparative procedure, small amount of racemization and high reactivity of the components. N,N'-dicyclohexylcarbodiimide (DCCI) is the most efficient coupling agent. The reaction mechanism is suggested (101)(34) as proceeding via the activation of the carboxyl group by addition of its proton to the double bond of the carbodiimide. The resulting compound then reacts either by O to N acyl migration to form an acyl urea or, in the presence of excess acid, by a second proton addition,

preventing O to N acyl migration and forming a symmetrical anhydride and a disubstituted urea. Formation of the peptide bond proceeds by reaction of an amino group with the anhydride.

Experimentally the amino and carboxyl components are mixed in relatively high concentration in organic solvent and cooled to $0^{o}C$. Dicyclohexylcarbodiimide is then added in portions over a time interval to produce a peptide product in excellent yield. Occasionally the N,N'-dicyclohexylurea by-product may cause difficulty during peptide isolation or crystallization, but this may be overcome by using diakylcarbodiimides which yield water soluble ureas on reaction.

Solid Phase Peptide Synthesis is a particular technique of peptide bond formation, developed by Merrifield in the early 1960's (102), that has become increasingly important in recent years for the formation of polypeptides. A resin, usually copolystyrene-2% divinylbenzene chloromethylated with chloromethylmethylether, is allowed to react with the triethylammonium salt of a CBZ or t-BOC amino acid. The resultant resin ester linked N-protected amino acid is analagous to the benzyl ester of the acyl amino acid, but is insoluble in commonly used solvents permitting the removal of reagents, by-products and impurities, solely by filtration. The N-protecting group is then removed from the amino acid by a normal cleaving agent and an activated N-protected amino acid added for the peptide forming step. After adequate reaction time the

reagents are removed by washing and the protective group cleavage and peptide forming steps repeated until the desired polypeptide product is obtained. The peptide may be liberated from the resin either by reaction with aqueous alkali in ethanol or by bubbling hydrogen bromide through a suspension of the resin in trifluoroacetic acid. It is also possible to cleave the peptide from the resin by ammonolysis. This elegant technique is extensively used for the synthesis of higher peptides.

3:4 THE SYNTHESIS OF PROTECTED AMINO ACIDS AND PROTECTED DIPEPTIDES

The choice of amino and carboxyl protecting groups had to be made bearing in mind the ease of synthesis of the protected amino acids and their stability to the condensation and equilibrium reactions, the specificity of the enzyme to the protected substrates, and the availability of reagents and labelled and unlabelled amino acids and dipeptides.

The synthesis of the dipeptide substrates had to involve as little handling of the material and be as efficient from a yield and optical purity point of view as possible. This meant that activating agents such as azide, acid chloride, mixed anhydride and active esters were less suitable than the condensing agent dicyclohexylcarbodiimide since they involved purification procedures and considerably more handling.

Consideration as to whether the condensation reaction should take place in free solution or in 'solid phase' suggested that although the ease of handling of materials favoured the solid phase peptide synthetic procedure, the additional work required in blocking the carboxyl group of the cleaved dipeptide and purifying the resultant product favoured reaction with pre-blocked amino acids in solution.

The specificity of thermolysin dictated that the amino acids be in the L-configuration at the sensitive site (103) and be hydrophobic with bulky side chains (104). Of the protected synthetic dipeptides used as substrates for thermolysin, the most rapidly hydrolysed were CBZ-gly-L-phe amide, CBZ-L-ala-L-leu hydrazide (94) and CBZ-L-thr-L-leu amide (79).

It was decided that the carbobenzoxy group would serve best in these studies as the amino protecting group since it was not susceptible to cleavage by the enzyme, was easily prepared, amenable to peptide synthesis via dicyclohexylcarbodiimide, and readily available from commercial sources.

The best carboxyl blocking group for these studies was the amide residue. It is a proven enzyme substrate and amide formation is relatively straight forward. Other advantages of this group over other carboxyl protecting residues are its positive contribution to the solubility of the dipeptide in aqueous solution, its non-susceptibility to enzyme cleavage or hydrolysis

(105) during peptide bond formation and its ready commercial availability.

Thus the dipeptides (and corresponding amino acids) chosen for this study were CBZ-gly-L-phe amide and CBZ-L-thr-L-leu amide.

Since amino acids may be purchased labelled with carbon14 only in the form of their free acids, the first necessity was to synthesize an N-protected labelled amino acid. From this product, in combination with an unlablled carboxyl protected amino acid, could be synthesized a labelled dipeptide. The production of both labelled amino acid and dipeptide meant that the equilibrium reaction could be studied from both directions enabling a more precise measurement of the equilibrium constant to be made.

The labelled amino acids prepared were CBZ glycine and CBZ-L-threonine. Only one labelled dipeptide was prepared, CBZ gly-L-phe amide.

a) *The Preparation of ^{14}C CBZ Glycine*

The carbobenzyloxylation of ^{14}C glycine was carried out using a modified method of the synthesis described by Bergmann and Zervas reported in Greenstein and Winitz (106). Two syntheses of ^{14}C CBZ glycine were performed.

563.0 mg (7.5 mm) of unlabelled glycine (Sigma London Chemical Co. Ltd) were dissolved in 2.5 ml (10 mm) 4N sodium hydroxide solution. An aliquot of this was carefully transferred by pasteur pipette into a phial containing 50 μCi of universally labelled ^{14}C glycine, specific activity 114 mCi/mm in 1.0 ml aqueous solution containing 2% ethanol (The Radiochemical Centre, Amersham). The solutions were mixed, carefully transferred to a 25 ml semi-micro flask and cooled to about 5°C. 3.0 ml (12 mm) 4N sodium hydroxide and 1.3 ml (9.1 mm) carbobenz oxy chloride (Sigma London Chemical Co. Ltd.) were added alternately to the reaction mixture in six equal portions over a period of 30 minutes, each sodium hydroxide addition being used to wash the radioactive phial prior to addition to the flask. The reaction mixture must be kept alkaline. The solution was stirred vigorously for 1 hour with cooling after the final addition of carbobenzoxy chloride, then transferred to a separating funnel and extracted with 2 x 5 ml diethyl ether to remove unreacted CBZ chloride. The solution was transferred to a clean semi-micro flask, cooled, stirred and acidified with 5N hydrochloric acid until a thick white precipitate of CBZ glycine formed and the solution was positive to Congo red. The mixture was stirred and cooled for a further 1 hour and then the precipitate filtered off under vacuum. The liquors were scavenged by the addition of more 5N hydrochloric acid, the precipitate filtered off, added to the original batch and the combined precipitates dried in a vacuum oven at 40°C for 18 hours.

Synthesis 1	Synthesis 2
Crude yield = 1.26 g (80.7%)	Crude yield=1.40 g (89.4%)

The crude CBZ glycine was recrystallized from boiling water, the recrystallization liquors being concentrated to provide a further crop of crystals.

Synthesis 1	Synthesis 2
Final yield = 0.96 g (61.2%)	Final yield = 1.10 g (70.1%)

The purity of the preparations was assessed using autoradiography and thin layer separation and counting.

10 x 20 cm glass plates were coated to a thickness of 0.25 mm with silica gel G (Merck Ltd., U.K. distributors, Anderman and Co. Ltd.) and activated by heating for 1 hour at 100°C. On cooling the plates were stored in a dessicator cabinet.

Three solutions were applied to a plate for radiochemical purity estimations: 20 µl of 5 mg/ml ^{14}C CBZ glycine preparation, 10 µl of 5 mg/ml CBZ glycine standard and 10 µl 5mg/ml glycine standard. The plate was developed in butanol/acetic acid/water, 48/12/20, v/v/v, and then dried in an oven at 100°C. The plate was autoradiographed for 24 hours using Ilford Industrial G type X-ray film. The film, upon development, displayed a single spot at Rf 0.63 (Fig 3:1). The radioactive strip on the plate was

covered and the remaining area of silica sprayed with chlorine-tolidene spray (87). Glycine Rf = 0.18, CBZ glycine Rf = 0.63. The radioactive strip was divided into 2 x 2 cm squares, the squares enclosing the areas corresponding to the standards spots, and the silica from each square scraped off into a scintillation vial. 5.0 ml Unisolve 1 scintillator (Koch-Light Laboratories Ltd.) and 2.0 ml water were added to each vial, the silica suspended in a thixotropic gel by vigorous shaking of the vial, and the samples counted on a Phillips Automatic Liquid Scintillation analyser.

The radiochemical purity is presented as the percentage of counts in the ^{14}C CBZ glycine square compared with the total counts of all the squares in the strip.

Synthesis 1	Synthesis 2
Radiochemical purity = 99.2%	Radiochemical purity = 99.5%

The specific activity of the ^{14}C CBZ glycine preparations was measured by counting at least two accurately weighed samples of each preparation dissolved in acetone in 5.0 ml Unisolve 1 scintillation fluid on the Phillips automatic counter. Acetone blanks were included to allow calculation for the quenching effect of the solvent. The efficiency of counting was measured by adding a known number of counts of a standardized ^{14}C n-hexadecane preparation (The Radiochemical Centre, Amersham) dissolved in Unisolve 1.

Synthesis 1	Synthesis 2
Specific activity = 15,281,090 dpm/mm	Specific Activity = 14,187,998 dpm/mm

b) *The Preparation of ^{14}C CBZ Threonine*. Carbobenzoxy-threonine was prepared using a modified Greenstein and Winitz procedure (106). Only one synthesis was performed.

836.6 mg (7.0 mm) L-threonine (Sigma London Chemical Co. Ltd) were dissolved in 10.0 ml distilled water and used to transfer 50 µCi of universally labelled ^{14}C L-threonine, specific activity 232 mCi/mm in 1.0 ml aqueous solution containing 2% ethanol (The Radiochemical Centre, Amersham), into a semi-micro flask. 2.1 g (25 mm) sodium hydrogen carbonate were added to the flask with a further 2.0 ml water. The solution was stirred vigorously at laboratory temperature and 1.5 ml (11 mm) carbobenzoxy chloride added in five portions over a period of thirty minutes. The stirring was continued for 2 hours after the final CBZ chloride addition. The solution was then transferred to a separating funnel and the unreacted CBZ chloride removed with 3 x 5 ml diethyl ether. The aqueous fraction was acidified to Congo red with 5N hydrochloric acid (approximately 5.0 ml) and the resulting oil extracted into 8 x 2 ml ethyl acetate and dried over anhydrous sodium sulphate. The ethyl acetate was then transferred to a round bottomed flask, the sodium sulphate washed with 2 x 2 ml dry ethyl acetate and the combined ethyl acetate fractions taken down to dryness under vacuum at 35°C. The resultant oil was dissolved in 5.0 ml dry ethyl acetate, gently heated and petrol ether 40-60 added until a white emulsion formed. More ethyl acetate was added until the solution was clear again, and it was

allowed to cool slowly. On further cooling in ice and with 'scratching' of the vessel wall, crystallization was induced. The crystals were centrifuged off on a bench centrifuge, washed with cold petrol ether 40-60, recentrifuged, and dried in a vacuum oven at 50°C.

Yield = 1.36 g (76.6%)

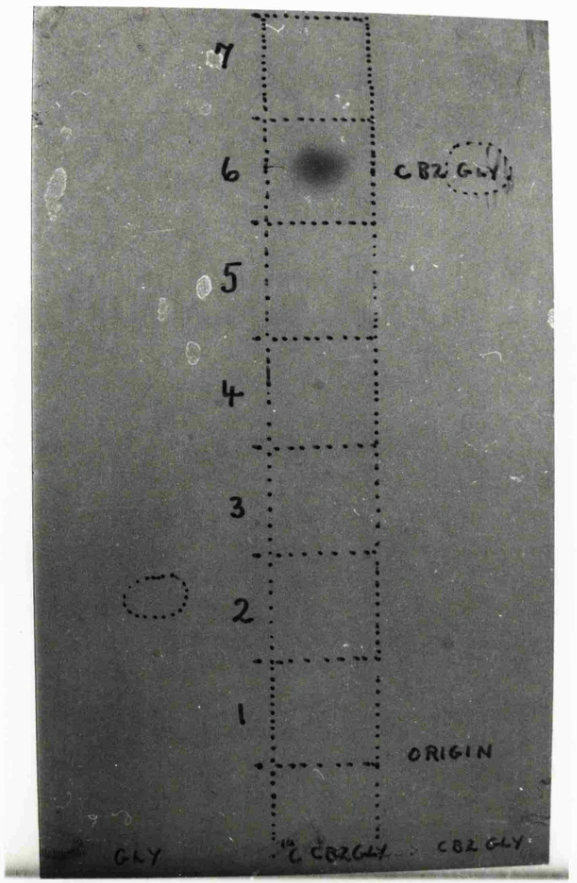
M Pt = 98 - 99.5°C Standard M Pt = 96 - 97.5°C

The radiochemical purity was assessed as before. A silica gel G plate was spotted with 50 µl of 3.5 mg/ml ^{14}C CBZ threonine, 10 µl of 10 mg/ml CBZ threonine and 10 µl of 10 mg/ml threonine, and developed in butanol/acetic acid/water, 48/12/20, v/v/v. The plate was dried and autoradiographed with Ilford Industrial G X-ray film for 24 hours. On development a single spot resulted at Rf 0.78 (Fig. 3:2). The radioactive strip was covered and the plate sprayed with chlorine-tolidene spray (87). Threonine Rf = 0.37, CBZ threonine, Rf = 0.78. The radioactive strip was divided into squares, the silica scraped off into scintillation vials and counted in 5 ml Unisolve 1 and 2 ml water on the Phillips scintillation counter.

Radiochemical purity = 98.6%

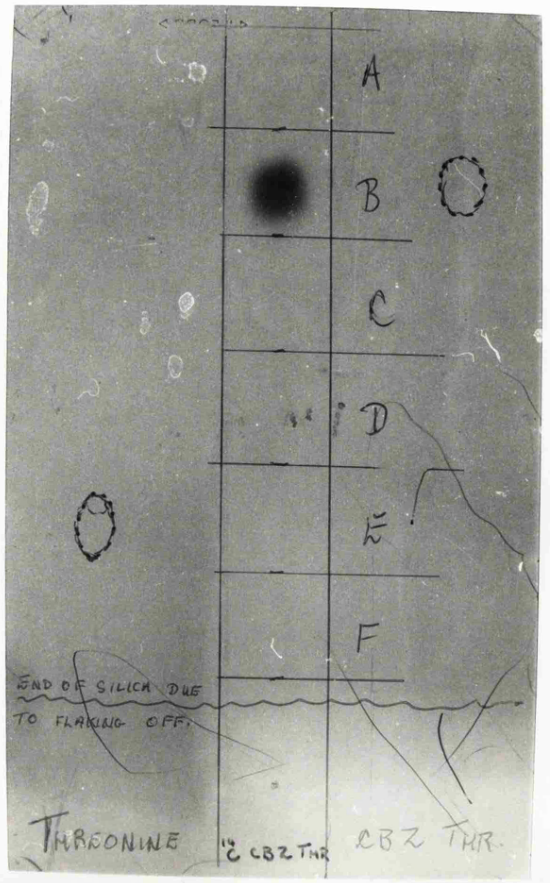
The specific activity was measured by accurately weighing two samples of the ^{14}C CBZ threonine, dissolving them in acetone,

Fig. 3:1.



Autoradiogram of CBZ glycine.

Fig. 3:2.



Autoradiogram of CBZ threonine.

and counting various volumes in 5 ml Unisolve 1.
The counting efficiency was assessed by the addition of ^{14}C n-hexadecane standard.

Specific activity = 15,957,435 dpm/mm.

c) *The Preparation of ^{14}C CBZ glycyl-L-phenylalanine amide*

The synthesis of CBZ-gly-phe-amide was adapted from the synthesis of CBZ-L-threonyl-D-alanine benzyl ester by Winitz et al. reported in Greenstein and Winitz (106) and the amidation of CBZ glycyl-L-phenylalanine ethyl ester by Fruton and Bergmann (107).

104.6 mg (0.5 mm) ^{14}C CBZ glycine and 107.8 mg (0.5 mm) L-phenylalanine methyl ester (Sigma London Chemical Co. Ltd.) were dissolved in 1.0 ml dichloromethane and approximately 70 µl (0.55 mm) triethylamine. 0.13 ml (0.5 mm) of dicyclohexylcarbodiimde (Eastman Organic Chemicals, U.K. distributors, Kodak Ltd.) was added and the solution thoroughly mixed and allowed to stand for 18 hours. The precipatate of dicyclohexylurea was removed by filtration and the solution washed with 2 x 2 ml water, 2 x 2 ml 0.1N hydrochloric acid, 2 x 2 ml water, 2 x 2 ml 5% sodium bicarbonate, 3 x 2 ml water, transferred to a clean tube with 2 x 1 ml dichloromethane and dried over anhydrous sodium sulphate for 3½ hours. The solution was then filtered, the sodium sulphate washed with 2 x 2 ml dry dichloromethane, and

the combined dichloromethane liquors taken down to dryness under vacuum at 38°C. The resulting oil was transferred to an ampoule with 2 x 1 ml dry dichloromethane and evaporated to dryness, the labelled CBZ glycyl-L-phenylalanine methyl ester oil now being ready for amidation.

Amidation was carried out in absolute methanol saturated with ammonia gas at 0°C.

Absolute methanol was produced by reaction with a Grignard compound as described in Vogel (108). 2.5 g of clean dry magnesium turnings and 0.25 g resublimed iodine were gently warmed with 30 ml methanol. As hydrogen evolution was slow a further 0.25 g iodine was added and the solution warmed until the iodine had disappeared and all the magnesium converted to the methoxide. 450 mls of methanol were added and the mixture boiled under reflux for 30 minutes. The product was then distilled over with the exclusion of moisture and stored in a stoppered vessel in a dessicator.

Ammonia saturated methanol was produced by passing ammonia gas from a cylinder (BDH chemicals Ltd.) through a drying column of calcium oxide, a guard tube and then into a tube containing approximately 5 ml absolute methanol in an ice bath.

1.0 ml ammonia saturated methanol was transferred to the precooled ampoule containing the ^{14}C CBZ glycyl-L-phenylalanine methyl ester, and the ampoule was sealed and left for 60 hours at laboratory temperature. The ampoule was then cooled and opened and the contents transferred to a round-bottomed flask and taken down to dryness under vacuum at 35°C. The resultant oil was transferred to a centrifuge tube with 2 x 1 ml methanol and crystallization induced by cooling and the addition of water. The product was recrystallized five times from methanol/water before being dried in a vacuum oven at 70°C.

Yield = 86.5 mg (50.2%)

M.Pt = 124°C Standard M.Pt = 122-123.5°C

Radiochemical purity was assessed using silica gel G.F. plates (Merck Ltd., U.K. distributors, Anderman and Co. Ltd.) spread to a thickness of 0.2 mm and activated 1 hour at 100°C, and Polygram Sil G/UV254 precoated plastic sheets (Mackerey-Nagel & Co.). Three solutions were applied to each chromatogram, ^{14}C CBZ glycyl-L-phenylalanine amide, CBZ glycyl-L-phenylalanine methyl ester, (Cyclo Chemical; U.K. distributors, Cambrian Chemicals Ltd.) and CBZ glycyl-L-phenylalanine amide (Sigma London Chemical Co. Ltd.) The chromatograms were developed in chloroform/methanol, 95/5, v/v, dried and visualized under U.V. ^{14}C CBZ-gly-phe-amide, Rf = 0.23, (plate), 0.28 (polygram); CBZ-gly-phe-amide, Rf = 0.23 (plate),0.29 (polygram): CBZ-gly-phe-methyl ester Rf = 0.93 (plate),

0.81 (polygram). The radioactive strips were divided into squares, the silica from the plate squares scraped off, and the polygram cut up, into scintillation vials and the material counted in 5 ml Unisolve 1 (and 2 ml water in the case of the silica powder) on the Phillips scintillation counter.

Radiochemical purity (silica plate) = 99.7%

Radiochemical purity (polygram) = 99.9%

The specific activity of the ^{14}C CBZ-gly-phe-amide was calculated from an accurately weighed sample dissolved in acetone. The counting efficiency was assessed by ^{14}C n-hexadecane standard addition.

Specific activity = 14,187,998 dpm/mm

The radioactive compounds were stored in crystalline form at $4^{o}C$.

CHAPTER 4

TECHNIQUES FOR THE MEASUREMENT OF THE EQUILIBRIUM CONSTANT

4:1 INTRODUCTION

The calculation of the free energy of peptide bond formation at elevated temperatures requires the determination of the equilibrium constant for the particular reaction under consideration from accurate measurements of the concentrations of reactants and products at equilibrium.

The equilibrium constant of a reaction may be defined by considering the dipeptide forming reaction represented by equation 4:1;

$$X + Y \underset{k_2}{\overset{k_1}{\rightleftharpoons}} X{-}Y + H_2O \qquad 4:1$$

where X and Y are amino and carboxyl protected amino acids respectively and X—Y is the protected dipeptide formed. The rate of the forward reaction is $k_1 (X)_a (Y)_a$ and the rate of the reverse reaction is $k_2 (X\text{-}Y)_a (H_2O)_a$ where k_1 and k_2 are the respective rate constants and $(X)_a$, $(Y)_a$, $(X\text{-}Y)_a$ and $(H_2O)_a$ are the activities of the reactants and products. At equilibrium the rate at which the reaction proceeds to the right is exactly balanced by the reverse reaction, i.e.

$$k_1 (X)_a (Y)_a = k_2 (X\text{-}Y)_a (H_2O)_a \qquad 4:2$$

and from the Law of Mass Action;

$$K = k_1/k_2 = \frac{(X\text{-}Y)_a (H_2O)_a}{(X)_a \quad (Y)_a} \qquad 4:3$$

where K is the equilibrium constant of the reaction and, by convention, the products of the reaction are placed in the numerator and the reactants in the denominator of the equation. In dilute solution the activities of the components may be replaced by their concentrations, the concentration of water, by convention, being unity, so:

$$K_{app} = \frac{(X\text{—}Y)}{(X)(Y)} \qquad 4:4$$

The apparent equilibrium constant, K_{app}, for a particular reaction may thus be determined by measuring the concentrations of the reactants and products at equilibrium and substituting them in equation 4:4

The change in the free energy (ΔG) of a chemical reaction is a measure of the amount of useful chemical work that may be derived from that particular reaction. The sign and magnitude of ΔG indicates the extent to which a reaction proceeds, a large negative ΔG characterising a reaction which goes almost to completion, a large positive ΔG indicating a reaction which only occurs to a very limited extent. The relationship between ΔG

and the equilibrium constant may be derived as follows:

$$X + Y \xrightarrow{\Delta G} X—Y + H_2O \qquad 4:5$$

where ΔG is a measure of the work that is gained by the reversible transfer of one mole each of X and Y from selected standard states to a large volume of reaction mixture in which X—Y and water are in equilibrium with X and Y, and the reversible transfer of one mole each of X—Y and water from the reaction mixture to selected standard states. The standard states are generally represented by either the pure solids X, Y, and X—Y and pure liquid water, or by aqueous solutions of X, Y, and X—Y at unit activity and pure liquid water. As pure solids the standard free energy change ΔG°_{s} is;

$$\Delta G^{\circ}_{s} = -RT \ln \frac{X_s}{X_a} + RT \ln \frac{Y_s}{Y_a} + RT \ln \frac{X\text{-}Y_a}{X\text{-}Y_s} + RT \ln \frac{H_2O_a}{1} \qquad 4:6$$

where the expressions X_s/X_a, Y_s/Y_a and $X\text{-}Y_a/X\text{-}Y_s$ are the activities of X, Y and X—Y in saturated solution, the activity of water being taken as unity, R is the gas constant and T the absolute temperature. As pure liquids the standard free energy change, ΔG^{o}, is;

$$\Delta G^{o} = -RT \ln \frac{1}{X_a} + RT \ln \frac{1}{Y_a} + RT \ln \frac{X\text{-}Y_a}{1} + RT \frac{H_2O_a}{1} \qquad 4:7$$

that is;

$$\Delta G^{o} = -RT \ln K \qquad 4:8$$

Equations 4:6 and 4:8 may be used to determine the standard free energy change in a chemical reaction from equilibrium data.

The value of the equilibrium constant of a chemical reaction depends upon the formulation of that reaction. The formulation can vary according to the ionization states of the reactants and products and thus K will vary depending upon the form in which the components are expressed. In the case of reactants and products as pure solids as their standard states there is only one formulation of the equation possible; amino acid (1) (solid) + amino acid (2) (solid) → Dipeptide (solid) + water (liquid) . For example:

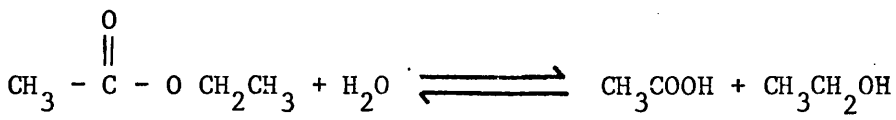
glycine (s) + glycine (s) → glycylglycine (s) + water(l)

$$\Delta G^{\circ}_{s} = +3230 \text{ cal/mole} \quad (45).$$

However, in the case of reactants and products in aqueous solution, several standard state equations may be written for a single reaction. In cases involving the gain or loss of a proton, as in many biological reactions, including the peptide synthesis and hydrolysis reactions under study in this work, there are three main ways, all correct, of expressing the reaction. The

three conventions may be illustrated by considering the reaction for the hydrolysis of ethyl acetate:

Convention I. The hydrolysis of ethyl acetate may be described by the equation:



The equilibrium constant would be:

$$K = \frac{(CH_3COOH)(CH_3CH_2OH)}{(CH_3COOCH_2CH_3)(H_2O)} = 18$$

$$\Delta G^{o} = -RT \ln 18 = -1710 \text{ cal/mole}$$

This Convention describes the reaction in non-ionic terms and results in a pH independent free energy change. The equilibrium constant is calculated on the basis of the components being in 1 molar aqueous solutions as the standard state and water at unit activity. In order to calculate the concentration of uncharged molecules in a solution at equilibrium a knowledge of the dissociation constants of the acids partaking in the reaction is required. For example, in the synthesis of glycylglycine (44), the four possible forms of the reactant, glycine are:

$\overset{+}{N}H_3CH_2COO^{-}$ ($^{+}G^{-}$); $NH_2CH_2COO^{-}$ (G^{-}); $\overset{+}{N}H_3CH_2COOH$ (^{+}G); NH_2CH_2COOH (G)

In order to determine the concentration of the uncharged molecule (G), the values for the ionization of glycine must be substituted in the following equations:

$$pH = pKa + \log (G^-) - \log (^+G^-)$$
$$= 9.7 + \log (G^-) - \log (^+G^-) \qquad 4:9$$

where 9.7 is the dissociation constant of the amino group when the carboxyl group is charged and:

$$pH = 4.0 + \log (G^-) - \log (G) \qquad 4:10$$

where 4.0 is the pKa of the carboxyl group when the amino group is uncharged. Similarly;

$$pH = 2.3 - \log (^+G) + \log (^+G^-) \qquad 4:11$$

and

$$pH = 8.0 - \log (^+G) + \log (G) \qquad 4:12$$

where 2.3 is the pKa of the carboxyl group when the amino group is charged and 8.0 is the pKa of the amino group when the carboxyl group is uncharged. From equations 4:9 and 4:10 or 4:11 and 4:12:

$$\log \frac{(^+G^-)}{(G)} = 5.7 \qquad 4:13$$

and similarly for the dipeptide (GG) and $(^+GG^-)$

$$\log \frac{(^+GG^-)}{(GG)} = 4.75 \qquad 4:14$$

Taking the peptide to amino acid ratio at equilibrium to be 0.001 (44), under standard conditions

$$K = \frac{(GG)}{(G)(G)} = 4,500 \qquad 4:15$$

Then for the reaction

$$G + G \longrightarrow GG + H_2O; \quad \Delta G^{o} = -4980 \text{ cal/mole} \qquad (44)(109)$$

This Convention has been extended by Carpenter (109) to provide the basis for his 'non-ionized compound convention', in which all the components are given in their unionized standard state and the formulation is used as the standard for the expression of pH independent values for K and ΔG^{o}. Although this Convention is convenient in the particular case of ester hydrolysis quoted by Carpenter, it is not generally widely used because dissociation constants, particularly of many physiologically important compounds, required for the calculation of the free acid concentration are not accurately known and, in particular, in reactions such as peptide synthesis it becomes difficult to use since there is no reasonable pH value at which the reactants and products, the amine and carboxyl groups, both exist in uncharged form. Convention I also suffers from the fact that it frequently does not accurately represent the actual reaction taking place in solution.

Convention II. Under physiological conditions (pH 7), the product of ethyl acetate hydrolysis is not acetic acid but acetate ion. Thus an alternative formulation of ethyl acetate hydrolysis is given by:

$$CH_3 - \overset{\overset{O}{\|}}{C} - OCH_2CH_3 + H_2O \rightleftharpoons CH_3COO^- + H^+ + CH_3CH_2OH$$

The equilibrium constant is:

$$K' = \frac{(CH_3COO^-)(H^+)(CH_3CH_2OH)}{(CH_3COOCH_2CH_3)}$$

$$= 18 \times 1.76 \times 10^{-5}$$

$$= 3.18 \times 10^{-4}$$

The value of K' is obtained from the value of K (in Convention I) and the dissociation constant for acetic acid, 1.76×10^{-5}. The standard free energy for this reaction is:

$$\Delta G^o = RT \ln 3.18 \times 10^{-4} = +4{,}900 \text{ cal/mole}$$

Thus both Conventions I and II give pH independent values for the free energy change and may be used to calculate the available free energy change at any pH or concentration by substitution of the correct activities (or concentrations) of the ionic species given in the equation which are present at the pH under consideration into the equation:

$$\Delta G = \Delta G^{o} + RT \ln \frac{(\text{Products})}{(\text{Reactants})}$$

Convention II is generally a more convenient convention for the expression of ΔG^{o} since its use at any pH requires only a knowledge of the pH and the pKa values associated with it. It is also a more realistic representation of the reaction which actually occurs in solution.

Convention III. The third Convention employs an equation which is formulated for a particular pH value, usually pH 7, and is expressed in terms of the total concentration of each reacting species.

$$CH_3 - \overset{\overset{O}{\|}}{C}-OCH_2CH_3 + H_2O \rightleftharpoons (CH_3COO^- + CH_3COOH) + CH_3CH_2OH + H^+ \ (pH)$$

The appropriate equilibrium constant is:

$$K'' = \frac{[(CH_3COO^-) + (CH_3COOH)]\ (CH_3CH_2OH)}{(CH_3COOCH_2CH_3)}$$

$$= K' \times 10^7 = 3.18 \times 10^3$$

K'' is the product of the equilibrium constant of Convention II and the hydrogen ion concentration.

$$\Delta G^{o'} = -RT \ln 3.18 \times 10^3 = -4{,}760 \text{ cal/mole}$$

Convention III represents compounds in their ionized form, the form in which most reagent molecules of biochemical interest are usually present under physiological conditions. Total concentration of components are employed in the derivation of K'' which yields a pH dependent standard free energy change, that is, ΔG^{o} is quoted for a defined pH. Convention III is probably the most generally used procedure for biochemical thermodynamic formulations (110). The effect of variations in pH on the standard free energy change calculated from Convention III may be derived mathematically. To evaluate these changes with pH, the difference in energy between the ionization states of the reactants and products at the two pH values is required. This may be expressed by the equation:

$$\Delta G^{o}_{2} = \Delta G^{o}_{1} + \Sigma \text{products } (Gi_2 - Gi_1) - \Sigma \text{ reactants } (Gi_2 - Gi_1) \qquad 4:16$$

where the subscripts 1 and 2 refer to the two pH values and ΔGi represents the free energy of ionization of the molecules at that pH. ΔGi at the two pH values is determined for each molecule in the reaction. ΔGi for any compound may be derived (109) by considering the change:

$$XHn \rightarrow XHn, XHn-1 \text{ ---------- } XH, X \qquad 4:17$$

where the right hand side of the equation represents an equilibrium mixture of the various forms of XHn So:

$$XHn = \Sigma Xi \qquad 4:18$$

The energy involved in this process is the difference in chemical potentials of the components on the two sides of the equation after equilibrium has been reached. This chemical potential is given by the expression:

$$-RT \ln C \qquad 4:19$$

where C is the concentration of the component involved. Thus

$$\Delta Gi = -RT \ln \Sigma Xi + RT \ln \Sigma XHn \qquad 4:20$$

or

$$\Delta Gi = -RT \ln \frac{\Sigma Xi}{XHn} \qquad 4:21$$

If K_1 K_2 etc, are the successive dissociation constants of protons from XHn, and H^+ the hydrogen ion concentration then:

$$XHn\text{-}1 = \left(\frac{K_1}{H^+}\right) XHn \qquad 4:22$$

and

$$XHn\text{-}2 = \left(\frac{K_2}{H^+}\right) XHn = \left(\frac{K_1}{H^+}\right)\left(\frac{K_2}{H^+}\right) XHn \qquad 4:23$$

then

$$\Sigma Xi = XHn\left[1 + \frac{K_1}{H^+} + \left(\frac{K_1}{H^+}\right)\left(\frac{K_2}{H^+}\right) + \text{---}\left(\frac{Kn}{H^+}\right)\right] \qquad 4:24$$

So equation 4:21 may be written

$$\Delta Gi = -RT \ln \frac{XHn}{XHn} \left[1 + \frac{K_1}{H^+} + \left(\frac{K_1}{H^+}\right)\left(\frac{K_2}{H^+}\right) + \text{-------} \left(\frac{Kn}{H^+}\right) \right] \qquad 4:25$$

or

$$\Delta Gi = -RT \ln \left[1 + \frac{K_1}{H^+} + \left(\frac{K_1}{H^+}\right)\left(\frac{K_2}{H^+}\right) + \text{------} \left(\frac{Kn}{H^+}\right) \right] \qquad 4:26$$

which is the general equation for ΔGi at any pH. By calculating ΔGi for each component of the reaction mixture and substituting the values in equation 4:16, the change in the free energy of a reaction system with pH may be determined.

These three Conventions outline the three most common methods of formulating thermodynamic equations. All are correct and all are useful. Other formulations derived from these are occasionally used, for example, the equation used by Morawetz and Otaki (111) to describe amide formation in aqueous media is a form of Convention II.

$$R_1COO^- + HN\begin{matrix}R_2\\R_3\end{matrix} \rightleftharpoons R_1CON\begin{matrix}R_2\\R_3\end{matrix} + OH^-$$

It was designed so that the charges on both sides of the equation balance and the carboxylate ion conversion to amide increases with the ratio of the concentrations of amine and hydroxide ion.

4:2 THE FORMULATION OF CBZ GLYCYL-L-PHENYLALANINE AMIDE AND CBZ-L-THREONYL-L-LEUCINE AMIDE FORMATION.

The reactions under study in this work may be represented by the synthesis of CBZ-glycyl-L-phenylalanine amide. CBZ-gly-phe-amide formation was formulated along the principles of Convention III.

Considering the reaction:

CBZ gly + L-phe amide $\rightleftharpoons$ CBZ-gly-phe-amide + water

in buffered solution at physiological pH, the equilibrium actually observed may be represented by the equation:

$$RCOOH + RCOO^- + \overset{+}{N}H_3R' + NH_2R' + H^+(pH) \rightleftharpoons 2RCONHR' + 2H_2O + H^+(pH)$$

$$\Delta G_1 = 0 \qquad 4:27$$

where the concentrations are in molarities and RCOOH represents CBZ glycine and NH_2R' represents L-phenylalanine amide. In this system there are also the independent equilibria:

$$RCOOH \rightleftharpoons RCOO^- + H^+ \ (pH) \qquad \Delta G_2 = 0 \qquad 4:28$$

and

$$\overset{+}{N}H_3R' \rightleftharpoons NH_2R' + H^+ \ (pH) \qquad \Delta G_3 = 0 \qquad 4:29$$

At physiological pH, CBZ glycine is fully ionized, so, subtracting equation 4:28 from the sum of equations 4:27 and 4:29:

$$RCOO^- + \overset{+}{N}H_3R' \rightleftharpoons RCONHR' + H_2O\ (liq) \quad \Delta G_4 = 0 \qquad 4:30$$

Therefore the synthetic reaction under study in this work in which the reactants and products are in their standard states is represented by equation 4:31.

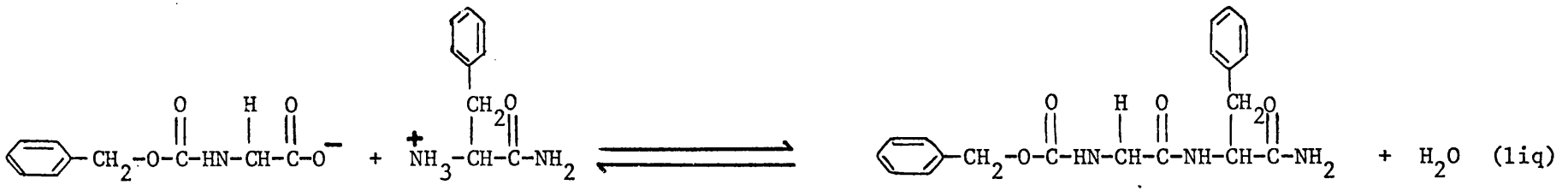
In order to determine the apparent equilibrium constant, and subsequently the standard free energy of dipeptide formation, it is necessary to measure the concentrations of the reacting species at equilibrium and then correct the figures for the proportion of the reactants which are unionized.

4:3 THE MEASUREMENT OF REACTANT AND PRODUCT CONCENTRATIONS AT EQUILIBRIUM

The measurement of reactant and product concentrations at equilibrium in an enzyme catalysed reaction may be carried out by either following the reaction to completion by the measurement of some physical or chemical parameter related to the appearance or disappearance of reactants or products during reaction, or by separating the components of the reaction mixture once equilibrium has been reached and measuring their concentrations as pure compounds.

Although the latter technique of concentration determination was eventually employed, the former method was investigated and two techniques of following the reaction tried:

Equation 4:31



CBZ glycine (1M) L-phenylalanine amide (1M) CBZ glycyl-L-phenylalanine amide (1M)

ΔG^{o} = to be measured

a) Titration assay. The hydrolysis of CBZ-gly-phe-amide at pH 8.0 proceeds with the liberation of a proton according to the equation:

$$R\text{-}CO\text{-}NH\text{-}R' + H_2O \rightleftharpoons R\text{-}COOH + NH_2\text{-}R' \quad 4:32$$
$$\downarrow\uparrow$$
$$RCOO^- + H^+$$

The ionization of CBZ glycine at pH 8.0 is practically 100%. By titrating the released proton with base both the rate and extent of reaction can be followed.

The apparatus employed was a Metrohm Combi-Titrator 3D (U.K. distributors: Shandon Southern Instruments Ltd.) used as a pH-stat. The electrodes were a Metrohm type EA 109 high temperature glass electrode and a Metrohm type EA 425 Ag/AgCl reference electrode, and the reaction was carried out at 60°C in a water-jacketed titration vessel.

20.0 ml of 5×10^{-3}M CBZ-gly-phe-amide in 0.01M KCl with 0.01M $CaCl_2$ was adjusted to pH 8.0 with base and 5.0 ml of approximately 2×10^{-5}M thermolysin solution added to start the reaction. Initially 5×10^{-2}M NaOH was used as titrant but gave poor rate data so was replced by 5×10^{-2}M KOH.

Rates of 0.3 µm CBZ-gly-phe-amide hydrolysed/min/mg enzyme were recorded.

The extent of hydrolysis could not be determined from the titration data, since in all the experiments carried out titrant in excess of that required to neutralise the equivalent of 100% hydrolysis of CBZ-gly-phe-amide was used. These results were probably due to several factors including; carbonic acid formation from CO_2 in the air dissolving in the reaction mixture, despite the titration vessel being closed to the atmosphere, possible autholysis of the enzyme and sluggish electrode response due to a protein coat forming on the glass membrane.

b) Spectroscopic Assay. In order to follow the reaction by spectroscopic means the reactants and products must differ in their absorption of light at a specific wavelength.

Initial sighting curves for both the dipeptides, CBZ-gly-phe-amide and CBZ-thr-leu-amide, and their component amino acids against water blanks carried out on a Unicam SP 800 recording spectrophotometer (Pye Unicam Ltd., Cambridge) indicated that the compounds only absorbed in the ultra-violet region of the spectrum and that there was no significant difference in absorbance between reactants and products at any specific wavelength. Detailed absorbance versus wavelength curves were plotted manually for CBZ-gly-phe-amide and its component amino acids at a concentration of 1×10^{-3} M against a water blank using a Unicam SP 500 spectrophotometer. These spectra are reproduced in figure 4:1 and confirm that there is no specific wavelength at which there is independent absorption by the amino acids and dipeptide.

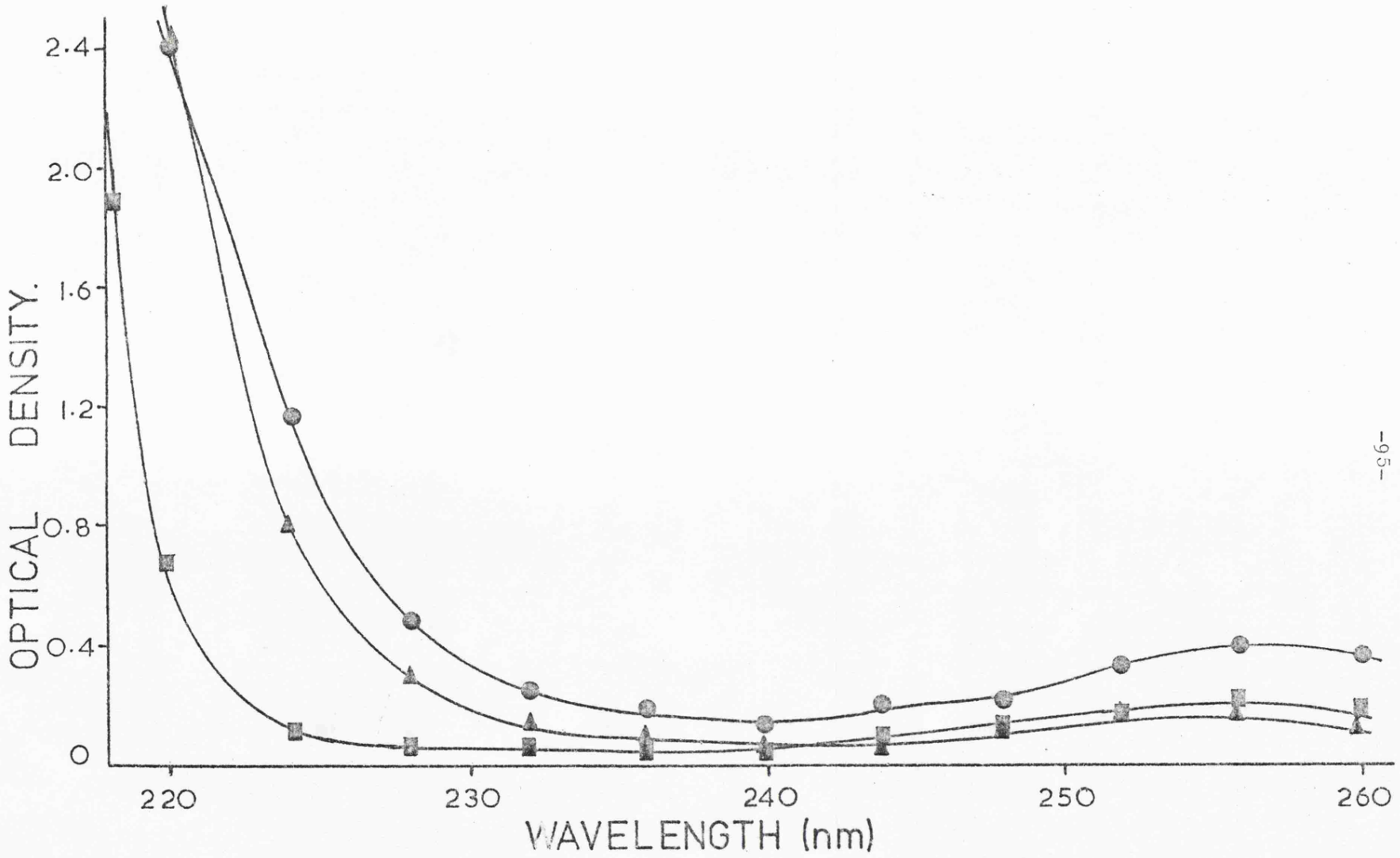


Fig. 4:1. Absorbtion Spectra of : CBZ gly. ■——■ Phe. amide. ▲——▲ CBZ-gly-phe-amide. ●——●

c) Concentration Measurements by Component Separation

In order to determine the most effective method of separating components in a mixture, it is necessary to consider in detail all the species present, their relative concentrations and their physical and chemical characteristics before designing a procedure for their separation.

Taking the enzyme catalysed formation of CBZ-gly-phe-amide as the typical reaction under study:

$$\text{CBZ gly + phe amide} \xrightleftharpoons{\text{thermolysin}} \text{CBZ-gly-phe-amide} + H_2O$$

the components present at equilibrium in such a system would be CBZ glycine, L-phenylalanine amide, CBZ-glycyl-L-phenylalanine amide, thermolysin, and components added to maintain the integrity and activity of the enzyme and the pH of the system, all in aqueous solution. The two systems used throughout these investigations to control pH and enzyme integrity were either 0.01M KCl with 0.01M $CaCl_2$, pH 8.0 (adjusted with KOH) or 0.01 M Tris/HCl buffer, pH 8.0, with 0.01M $CaCl_2$, the calcium chloride in both systems being required for enzyme activity. The relative concentrations of the reaction system components were estimated from the experiments of Dobry et al. (4) who, using a similar equilibrium system, found a 1.2% synthesis of dipeptide. As this value was the highest percentage synthesis of dipeptide in dilute aqueous solution recorded in the literature (see Table 1:1, cf ΔG^o values) an arbitrary figure of 1% dipeptide

synthesis was used for these estimations. The optimum enzyme concentration, from preliminary studies, was taken to be 2.5×10^{-6}M. Having regard to the limited solubility of the dipeptide in aqueous solution, the initial concentrations of the reactant amino acids were taken as 5×10^{-3}M. Thus, the reaction system at equilibrium was estimated as being:

CBZ glycine, 5×10^{-3}M
Phenylalanine amide 5×10^{-3} M
CBZ-gly-phe-amide 5×10^{-5}M
Thermolysin 2.5×10^{-6}M

} in 1×10^{-2}M buffer with 1×10^{-2} M $CaCl_2$

The stability of the compounds in solution and their molecular properties also must be known so that the most appropriate separation technique may be applied. The stability of the reaction mixture components with respect to pH, solvent and temperature were considered. All the molecules, with the exception of the enzyme, were stable over a fairly broad pH range although the degree of ionization of the amino acids and buffer components would vary according to the pH, At pH values below pH 5 and above pH 9 the enzyme was irreversibly inactivated (78). Solvent effects on the stability of the reaction mixture molecules were negligible although it was noted that the dipeptide was only slightly soluble in aqueous solution and that the buffer and calcium chloride components were relatively insoluble in acetone and ethanol. The

enzyme was reported to be precipated by cold acetone and unstable in alcohol (79). The amino acids, dipeptide and buffer constituents were stable over a broad temperature range, as was the enzyme, but only over a limited period of time. The dipeptide and enzyme were unstable to a combination of elevated temperatures and low pH values.

The molecular properties of the components were considered according to their size, ionic properties and polarities. The molecular size of a compound is related to its molecular weight. Generally, the larger the molecular weight the larger the molecule, although this is not true in all cases. However in order to compare the molecules in this particular case, molecular weight was used as the basis of molecular size. The smallest molecules were the buffer and calcium chloride components, the molecular weight of KCl being 74, Tris 121, and $CaCl_2$ 111. The amino acids were next in size, CBZ gly 209 and phe amide 164, followed by the dipeptide at a molecular weight of 355. The enzyme had a molecular weight of 34,700 (89).

Separations on a basis of molecular size are usually achieved either by dialysis, ultrafiltration or gel filtration. In the cases of dialysis and ultrafiltration a membrane is used which has pores of sufficient size to allow the smaller molecules to pass, but inhibits the passage of the larger molecules. However, the molecules of particular importance in this study, the dipeptide and amino acids,

do not differ sufficienty in size from each other, or from the buffer components, to be separated by these techniques. The separation of the amino acids and dipeptide by gel filtration, that is, by passage down a column containing a swollen gel such that the smaller molecules permeate the gel matrix and are retarded whereas the larger molecules are excluded and are not retarded is possible, particularly if interfering molecules, such as the enzyme and buffer components are removed prior to gel filtration by precipitation or by the selective solution of the amino acids and dipeptide.

The ionic properties of the molecules in the reaction mixture are of prime importance, as this is where the most significant difference between the groups of molecules exist. The extent of ionization of the molecules varies from complete ionization in the case of potassium and calcium chlorides to the completely uncharged molecule of CBZ-gly-phe-amide. At physiological pH values the Tris molecule is negatively charged, as is the amino acid phenylalanine amide, whereas CBZ glycine is positively charged. The enzyme, based on the evidence of its primary structure (89) contains both positive and negative charges. The extent to which the amino acids and Tris molecules are ionized in aqueous solution depends upon the pH of the solution and the ionization constants of the molecules. At physiological pH values (around pH 7-8), CBZ glycine is fully ionized and L-phenylalanine amide is approximately 50% ionized, as is Tris. The enzyme, which contains numerous ionizable groups is charged at most pH values. Grading the molecules of the reaction

mixture according to charge density, CBZ-gly-phe-amide is uncharged, phe amide and Tris molecules are 50% charged and CBZ gly, KCl and $CaCl_2$ are 100% charged. The enzyme, because of its numerous ionizable groups may also be regarded as being fully charged.

Separation techniques utilizing the charge characteristics of a molecule to promote its separation include ion exchange chromatography, electrophoresis and solvent extraction. Ion exchange chromatography is based on the adsorption and elution of reaction mixture molecules from the ion exchange substances as a function of pH. Thus in a mixed bed column, which contains both anionic and cationic resins, at neutral pH, in theory the uncharged dipeptide molecule would be the only molecule eluted. Upon elution with base, the phe amide and Tris fractions would become uncharged and released from the resin, whilst acid elution would release the CBZ gly fraction. The enzyme would probably remain bound. Electrophoresis separations depend upon the migration of a substance under the influence of an electric field. Positively charged molecules migrate to the cathode and negatively charged molecules to the anode. The separation is usually carried out on an inert medium, such as cellulose (paper) or silica gel, which is soaked with buffer and to which the sample to be separated is applied. A voltage is introduced via electrodes across the supporting medium, the buffer carrying the current, and the charged ions migrate towards their oppositely charged electrode. In the case of the reaction system considered here, at a pH value of about 5, the amino acids will be fully charged, the dipeptide

uncharged and the enzyme partially charged. After passing a potential for a suitable length of time across a supporting medium to which the reaction mixture has been applied, and choosing a visualizing method that is specific for amino acids and their derivatives, the degree of separation of the amino acids and enzyme from the dipeptide may be observed. Solvent extraction as a means of separating the reaction mixture components on the basis of their charge differences is possible and depends upon the differential solubility of the components in a two phase solvent system. The system, however, is often difficult to develop and lengthy to carry out.

The polarity of the reaction mixture compounds is also important in the development of a separation technique. The polarity of a molecule is related to its ionic properties in that highly polar molecules have a high dielectric constant and are thus capable of entering into hydrogen bond formation whereas nonpolar molecules are incapable of hydrogen bond formation and have low dielectric constants. These properties result in the fact that non-polar substrances generally dissolve in non-polar organic solvents whereas polar substances dissolve in polar solvents, such as water.

Separation techniques utilizing this property include solvent extraction, paper and thin layer chromatography, and precipitation and crystallization methods. Solvent extraction, as mentioned previously, is not frequently used as a separation technique unless the molecules to be separated differ considerably in their dielectric

constants so that the molecules of interest may be partitioned into one liquid phase whilst leaving the other substances dissolved in the second liquid phase. Since the molecules of interest in the reaction mixture, i.e. the amino acids and dipeptide, do not differ sufficiently in their solubilities in polar and nonpolar solvents for easy separation, and since separation by a solvent extraction technique, such as counter-current distribution is lengthy and tedious to carry out to completion, this technique was not considered for this study. Paper and thin-layer chromatography utilise the differential partition ratios of the substances to be separated between a mobile liquid phase and a solid, or liquid, stationary phase, as a means of isolating the compound of interest. The stationary phase is usually cotton cellulose in the case of paper chromatography and silica or alumina gel in the case of thin-layer chromatography with either the dry adsorbant or the water absorbed by the adsorbant, acting as the partioning phase. The mobile liquid phase is chosen so that its composition excentuates the differences between the molecules to be separated by the greatest amount. Application of the sample to the base of the chromatogram and its development in a saturated atmosphere of the mobile liquid phase is straightforward. The visualization of the compounds on the resultant chromatogram may occasionally be difficult and chromatograms are limited in the quantity of material that may be applied and in the accuracy of concentration determinations in a quantitative separation. Precipitation and crystallization techniques are based on the selective solubility, or insolubility, of the chemicals to be separated, in a particular solvent system. The criterion

which must be satisfied for precipitation of a compound to occur is that the solution must become supersaturated with respect to that particular solute, which may be achieved either by direct chemical action which forms a new chemical species that is insoluble in solution or by producing changes in the system which decrease the solubility of the solute in the parent solution. Various factors determine the solubility of solid solutes including temperature, particle size, degree of supersaturation, solvent-solute interactions and variations in pH and salt concentration. In considering the species to be separated in the reaction mixture under investigation, in particular the separation of the dipeptide from the amino acids, temperature, particle size and pH and salt concentration variations have little effect since the molecules are very similar. However considering the difference in polarity between the polar amino acids and the nonpolar dipeptide, variation of the solvent - solute interactions by addition of a suitable reagent to the reaction mixture should produce supersaturation of the dipeptide and its precipitation from solution.

Of the separation techniques discussed, ion-exchange chromatography, paper and thin-layer chromatography and electrophoresis, and precipitation/crystallization methods were tried as means of obtaining a pure dipeptide product in quantitative yield.

(i) *Ion-exchange Chromatography*

In order to isolate the uncharged dipeptide fraction from a

reaction mixture containing anionic and cationic molecules, both anionic and cationic ion - exchangers must be employed. Substituted Sephadex G-25 ion-exchangers (Pharmacia Ltd.) were chosen since they combined a high degree of cross-linking, which encourages the penetration of small molecules, with the advantages of a polysaccharide based structure, such as gel filtration properties and product stabilization. The two ion-exchangers, DEAE Sephadex A-25 and CM Sephadex C-25, could not be prepared as a mixed bed column as the two oppositely charged fractions combined to form a homogeneous swollen polysaccharide gel. A 'split level' column had to be prepared using the anion exchanger in the lower half and the cation exchanger in the upper half and separating them with a 10 μ bed support net (Pharmacia Ltd.). Two sizes of column were used in this investigation, a 1 x 15 cm water jacketed column made by Bath University glass-blower, and a 1.6 x 40 cm water jacketed column from Pharmacia Ltd.

The elution buffers were chosen so that the first solution used was at neutral pH to elute uncharged and weakly charged molecules, the second solution basic to release the cationic molecules and the third solution acidic to release the anions. The eluting solutions were required not to absorb in the ultra violet region of the spectrum to enable detection of the components in the eluate by U.V. The eluate was collected in 1 ml fractions on a L.K.B. Ultrorac Fraction Collector, (L.K.B. Ltd., Sweden), and the components in this eluate detected by measuring the optical density

of the solutions on a Pye Unicam S.P. 500 spectrophotometer at 218 nm. The concentration of dipeptide in the fractions was determined in two ways. A calibration curve of dipeptide concentration against optical density was constructed for both dipeptides (CBZ-gly-phe-amide and CBZ-thr-leu-amide) and the concentration of dipeptide in each column fraction measured and the results summed for the total dipeptide fractions. The dipeptide concentration was also assessed by constructing an elution diagram from the column fractions, measuring the area under the dipeptide peak by means of an automatic area meter (Hayashi Denko Co. Ltd., Tokyo). and comparing it to a series of peak areas derived from eluting various concentrations of dipeptide through the column.

The columns were first calibrated with respect to the dipeptides, and amino acids and enzyme and their respective eluting solutions, to determine the rates and volumes of elution. The dipeptides, CBZ-gly-phe-amide and CBZ-thr-leu-amide, at concentrations from 2.5×10^{-4}M to 2.5×10^{-5}M in water were eluted through the columns at 60°C with water. It was noted that solutions of dipeptide in both 0.01M Tris/HCl buffer, pH 8.0 with 0.01M $CaCl_2$, and 0.01M KCl with 0.01M $CaCl_2$ adjusted to pH 8.0 with KOH, were retained in the column longer and required almost double the amount of water to elute them. The phenylalanine and leucine amides at concentrations of 1×10^{-3}M and 5×10^{-3}M in water were not eluted with water at 60°C. 0.01M NaOH was used to release the amides from the column. It was found that solutions of the amides in Tris/HCl buffer were

eluted with water at 60°C, the buffer having a neutralizing effect on the ionic properties of the exchangers. However, 0.01M KCl with 0.01M $CaCl_2$ did not have this effect and the amides were not eluted until the addition of NaOH. As a result all dipeptide forming reactions were carried out in KCl/$CaCl_2$ solution.

CBZ glycine and CBZ threonine at concentrations of 1×10^{-3}M and 5×10^{-3}M in water and KCl/$CaCl_2$ were not eluted from the columns at 60°C with water. The protected amino acids could only be removed from the exchangers with 0.01M HCl if they had been originally in solution in water.

The enzyme, thermolysin, applied to the column at a concentration of 0.2 mg/ml (approximately 6×10^{-6}M) in water, Tris/HCl or KCl/$CaCl_2$, could not be removed from the exchangers with water, 0.01M NaOH or 0.01M HCl.

Prior to separating a reaction mixture on the columns an artificial reaction mixture was prepared consisting of:

4×10^{-5}M	CBZ-gly-phe-amide
5×10^{-3}M	CBZ gly
5×10^{-3}M	Phe amide
0.2 mg/ml	Thermolysin

in 0.01M KCl with 0.01M $CaCl_2$ adjusted to pH 8.0 with KOH. The 1 x 15 cm column and the artificial reaction mixture were preheated

to 60°C and 1.0 ml of the mixture applied to the column and eluted with water, 0.01M NaOH and 0.01M HCl respectively. The resultant elution diagram is presented in Fig 4:2. The size of the peak eluted by the 0.01M NaOH cannot be totally accounted for by the concentration of phenylalanine amide. It was suspected that the other amino acid and/or the enzyme was eluted with the phenylalanine amide and contributed to the peak.

The experimental reaction system consisted of:-

5×10^{-3}M	CBZ gly
5×10^{-3}M	Phe amide
0.2 mg/ml	Thermolysin.

in 25.0 ml of 0.01M KCl with 0.01M $CaCl_2$, adjusted to pH 8.0 with KOH. The reaction was carried out for either one or two hours at 60°C, the pH of the system being maintained at pH 8.0 by the addition of 0.01M NaOH. After the requisite time, 1.0 ml of reaction mixture was removed and applied to a freshly prepared 1 x 15 cm column of A25/C25 Sephadex and eluted with water at 60°C. A typical elution diagram is illustrated in figure 4:3. The usual small dipeptide peak at the beginning of the diagram is swamped by a large second peak. Analysis of the fractions from these and other similar peaks from other column separations, using thin layer chromatrography on silica gel G developed in butanol/ acetic acid/water, 48/12/20, v/v/v, and visualized with ninhydrin and chlorine/tolidene sprays (Figs. 4:4 A and B) showed the small

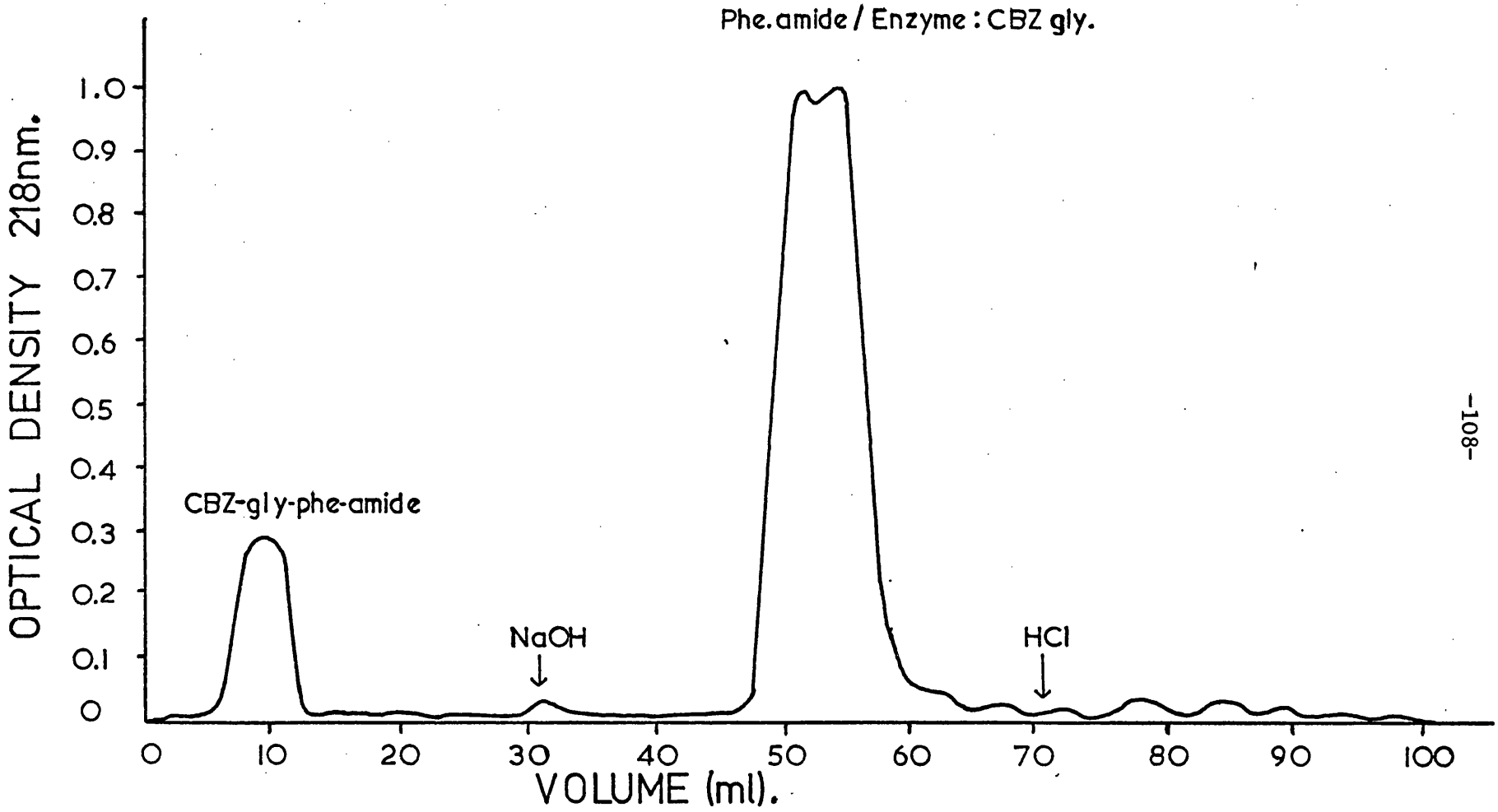


Fig. 4:2. Elution Diagram of the Artifical Reaction Mixture.

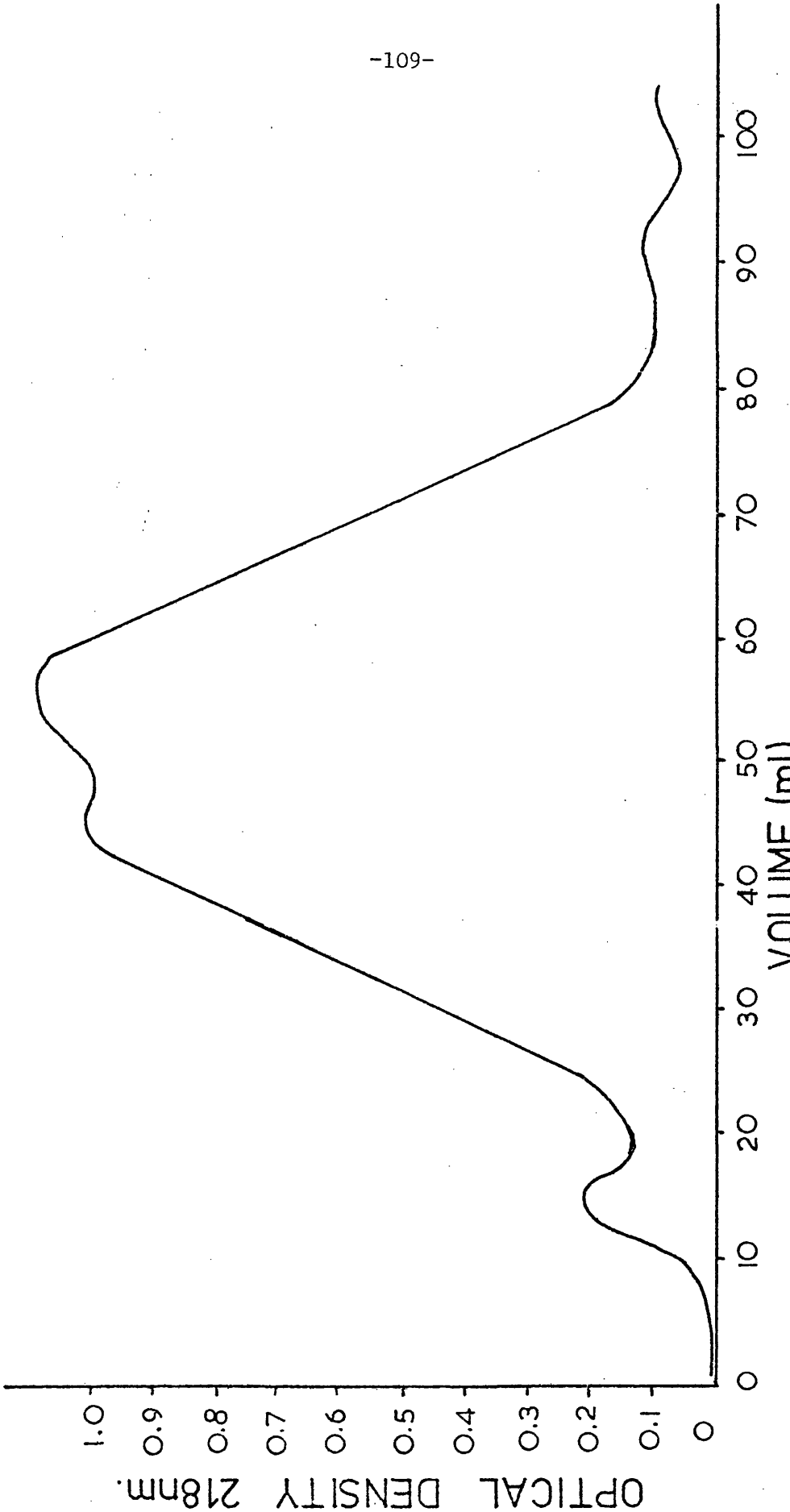
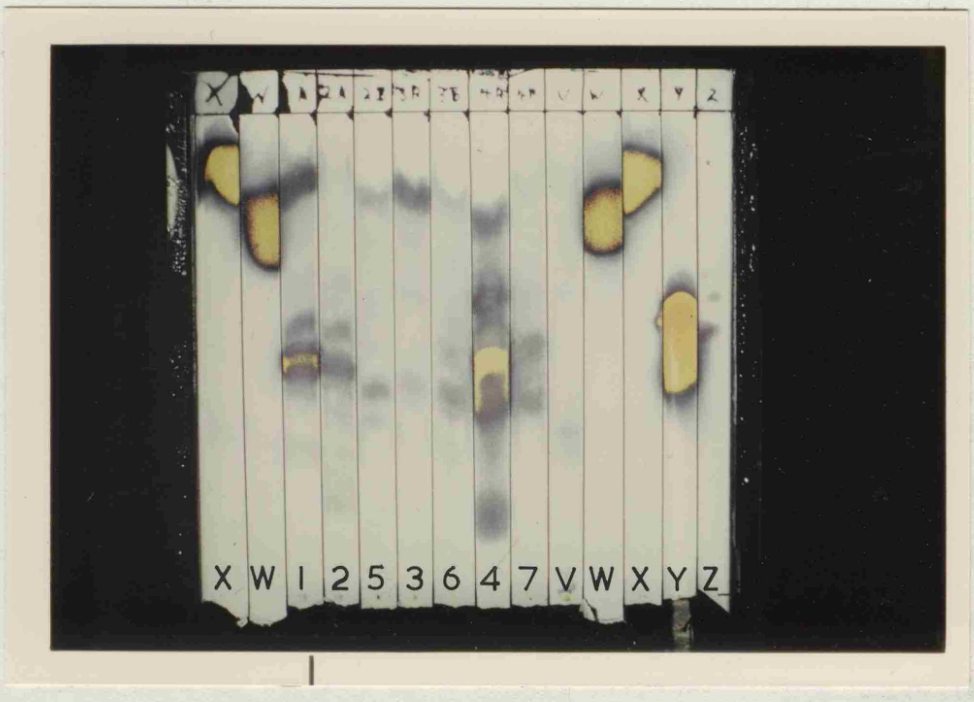


Fig. 4:3. Elution Diagram of the Reaction Mixture.

Figs. 4:4 A & B. The Analysis of Ion-exchange Column Fractions by T.L.C.

Fig. 4:4A. Ninhydrin Spray.

Fig. 4:4B. Chlorine/tolidene Spray.



V = Enzyme. W = CBZ-gly-phe-amide.
X = CBZ gly. Y = L-phe amide.
Z = L-phenylalanine.
1,2,3,4 = Dipeptide peaks. 5,6,7 = Large peaks.

dipeptide peaks, (1, 2, 3 and 4, Fig. 4:4) to consist of a mixture of dipeptide and amino acid, and the large peaks (5, 6 , and 7, Fig 4:4) to contain phenylalanine amide and other components. The poor separation and premature appearance of the phenylalanine amide peak was attributed to an interaction between the ion exchangers, the reaction mixture constituents and the $KCl/CaCl_2$ and NaOH buffer components.

The column separation was repeated many times altering a variety of factors. In order to remove enzyme interference 20 ml of reaction mixture, upon completion of reaction, was pipetted into 100 ml ice cold ethanol to precipitate the catalyst. The optical density of the solution was measured before and after the solution had been filtered through a 0.2μ pore size Metricel filter (Gelman Hawksley, Ltd, Sussex) under vacuum. No difference in optical density readings between filtered and unfiltered solutions was observed, suggesting ethanol was not a good precipitation agent for the enzyme. Precipitation of the enzyme in the ice-cold acetone and filtration through Millipore 'fluoropore' filters (Millipore Ltd., London) did not improve the column separation characteristics significantly. A larger column was tried, 1.6 x 40 cm, the reaction mixture first being pipetted into ice cold acetoneto stop the reaction, the acetone solution taken down to dryness on a rotary evaporator then dissolved in 10.0 ml hot water and either 5 ml or 10 ml applied to the

column and eluted with water at either 60°C or laboratory temperature. Smaller volumes could not be applied to the column since diffusion and dilution within the column made the dipeptide too dilute to be detected upon elution. The resolution of the mixture was not improved by the use of a larger column. Different exchangers were tried. A mixed bed of Zerolit 225 SRC 13 standard resin and Deacidite FF-1P SRA 65 standard resin (BDH Ltd.) in a 1.8 x 36 cm column did not separate the reaction mixture components.

The difficulty in separating the dipeptide from the other components, particularly the partially charged phenylalanine amide, resulted in ion-exchange chromatography being discarded as a useful technique for the separation of the reaction mixture products.

As column chromatography with ion-exchange materials proved unsatisfactory, gel filtration chromatography was tried. A 1.8 x 36 cm column was filled with Biogel P2 (Bio-rad Labs) and calibrated with a 2×10^{-4}M aqueous solution of CBZ-gly-phe-amide. However the dipeptide was retained by the gel to an excessive extent not appearing in the column effluent until 80 ml had been eluted (approximately 16 column volumes), thus making it unsuitable for the separation of reaction mixture components.

(ii) *Paper and Thin-Layer Chromatography and Electrophoresis*

The separation of the reaction mixture components by paper and thin-layer chromatography was difficult due to the similar adsorption and partition properties of the dipeptide and CBZ amino acid. Typical chromatograms are illustrated in Figures 4:4 and 4:5. Figure 4:5A and B illustrates the catalysis of the reaction by thermolysin from both the amino acid and the dipeptide sides of the equation. Both figures confirm that a quantitative separation of the dipeptide from amino acids on such a system is not feasible.

The electrophoretic separation of reaction mixture components was initially tried on Whatman 3MM paper. Amino acid standards and a 100 µl aliquot of a concentrated reaction mixture solution were applied along the middle of the paper, although difficulty was experienced in applying such a relatively large volume of reaction mixture to paper. The paper was soaked in water/acetic acid/pyridine buffer, 980/9.5/20, v/v/v, at pH 5.4 and the electrophoresis carried out at a constant current of 100 mA for 45 minutes. After drying the paper was visualized with chlorine/tolidene spray. However, due to excessive loading of the paper, no clear separation of components could be detected. To increase the capacity of the electrophoretogram, thin layer electrophoresis was tried. 1.0 mm thick silica gel G thin layer plates were prepared and a series

Figs. 4:5 A & B. The Attempted Separation of Amino Acids and Dipeptide on T.L.C.

Fig. 4:5A. Ninhydrin Spray

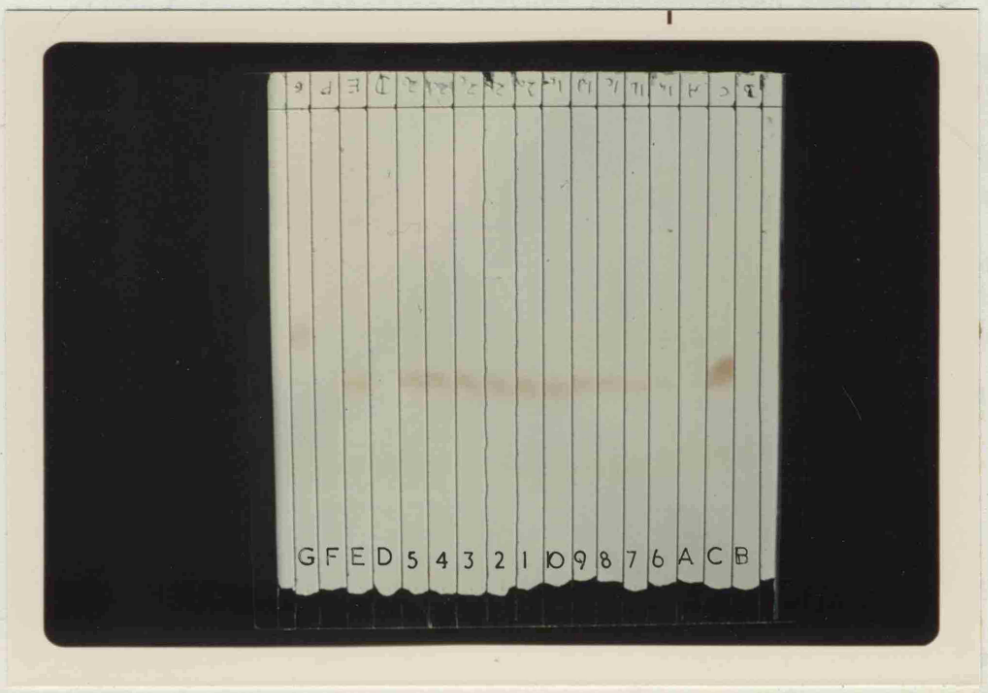
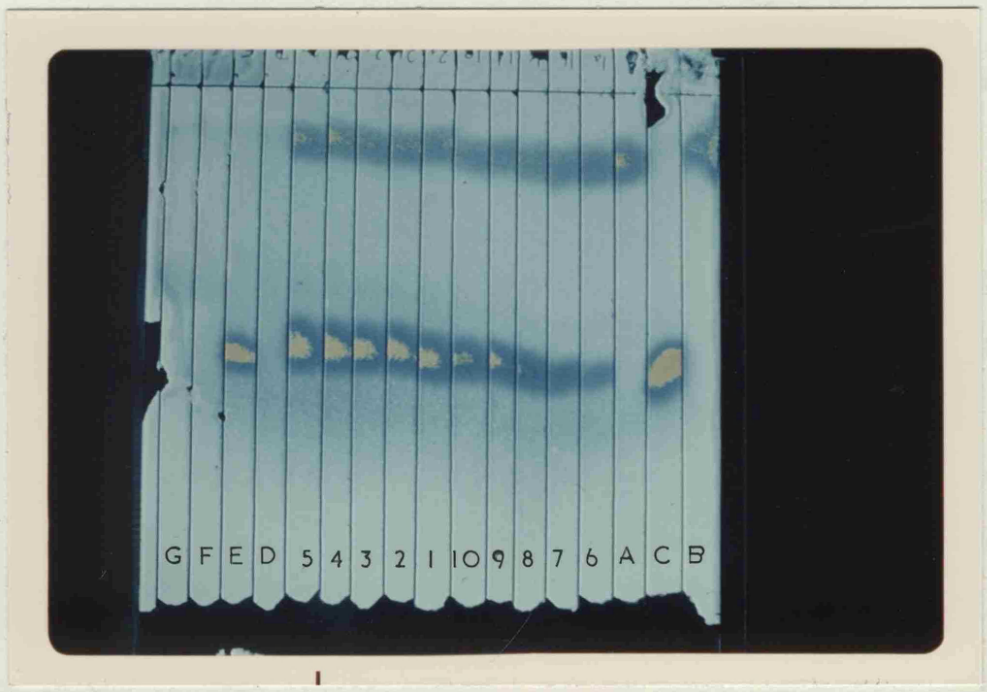


Fig. 4:5B. Chlorine/tolidene Spray.

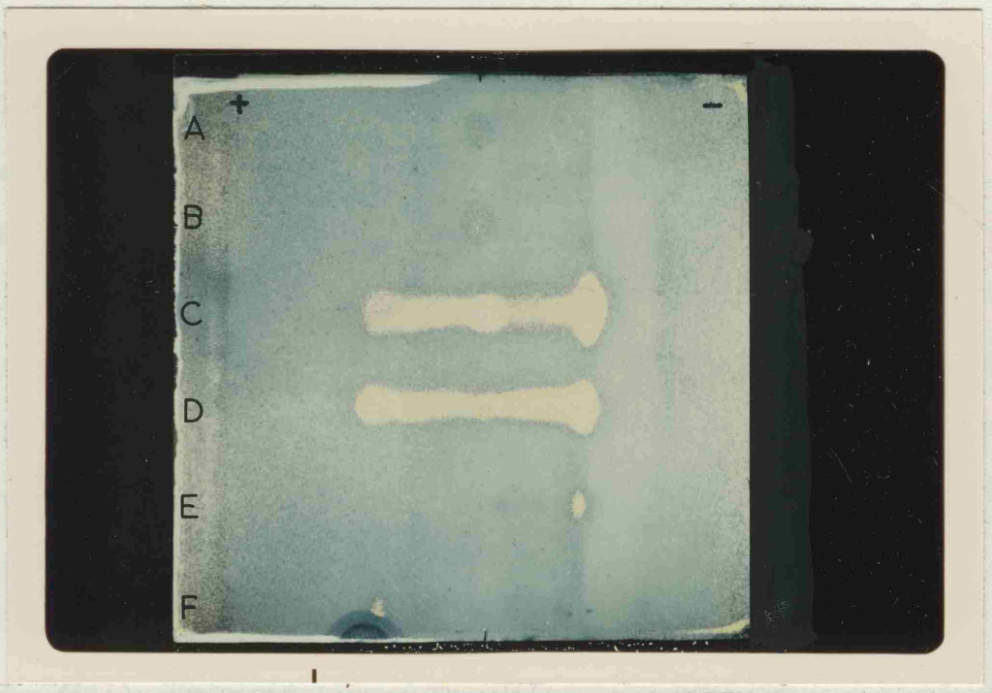


Chromatograms developed in butanol/acetic acid/water, 118/12/20, v/v/v.

A = CBZ-gly-phe-amide.
B = CBZ gly.
C = L-phe amide.
D = Enzyme.
E = Gly-phe.
F = Gly.
G = L-phe.
1 - 5 = Synthesis reaction.
6 - 10 = Hydrolysis reaction.

of standards, an artificially prepared reaction mixture and an aliquot from a reaction mixture concentrated from 20 ml to 2 ml on a rotary evaporator, applied along the middle of the plate. The plate was placed in a Shandon thin -layer electrophoresis tank and kept cool by water circulating through the cooling platten from a Churchill Chiller Thermo Circulator. The plate was soaked with water/acetic acid/pyridine buffer, 980/9.8/20, v/v/v, pH 5.4, via the wicks from the electrode compartments and a constant current of 100 mA maintained for 1 hour during the electrophoretic run. After drying the plate was visualized with chlorine/tolidene spray and is illustrated in figure 4:6. In order to improve the separation of the amino acids from the dipeptide, the electrophoretogram was repeated at a constant current of 100 mA for 2 hours (Figure 4:7). Although the amino acids had migrated further from the middle of the plate, there was still considerable 'tailing' to the centre. To eliminate the 'tailing' and isolate the dipeptide fraction, a combined chromatography and electrophoresis technique was employed. A 100 µl sample of the concentrated reaction mixture was applied to the bottom of a 1 mm thick silica gel G T.L.C. plate and developed in butanol/acetic acid/ water/pyridine, 90/4.5/18/10, v/v/v/v, pH 5.4. After completion of the chromatographic run, the plate was dried and then placed in the Shandon T.L.E. tank and soaked with water/acetic acid/ pyridine buffer, 980/9.8/18, v/v/v, pH 5.4, via the wicks. The electrophoresis was run for 2 hours at a constant current

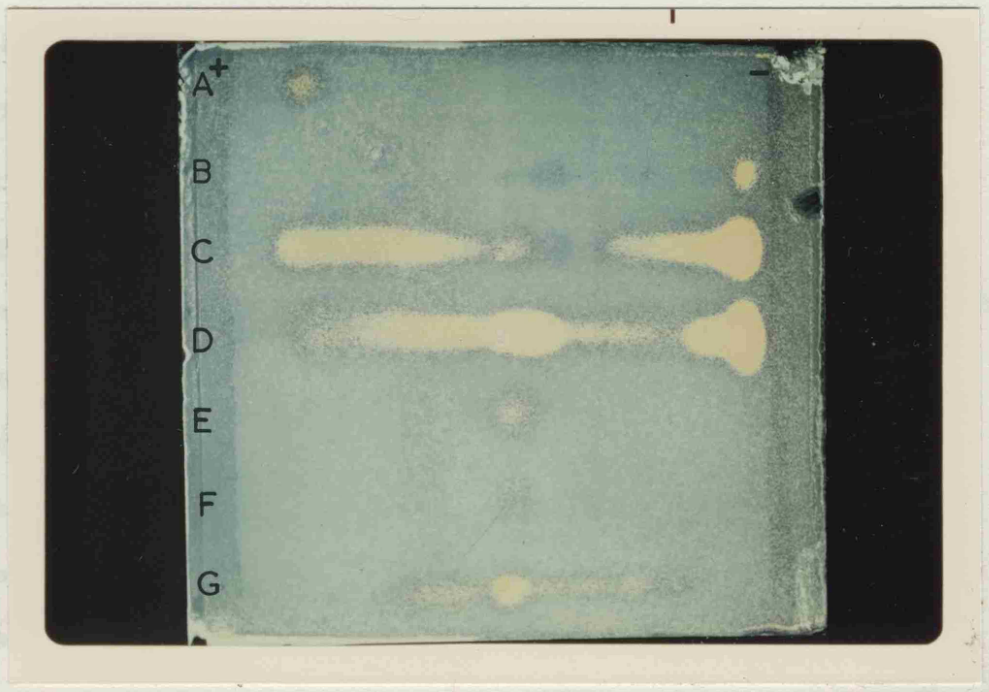
Fig. 4:6. Reaction Mixture Resolution by Electrophoresis, 1 hr.



A & B = CBZ-gly-phe-amide. C & D = Reaction mixture.

E = Phe. amide. F = CBZ gly.

Fig. 4:7. Reaction Mixture Resolution by Electrophoresis, 2 hrs.



A = CBZ gly. B = Phe. amide.

C & D = Reaction mixture. E & F = CBZ-gly-phe-amide.

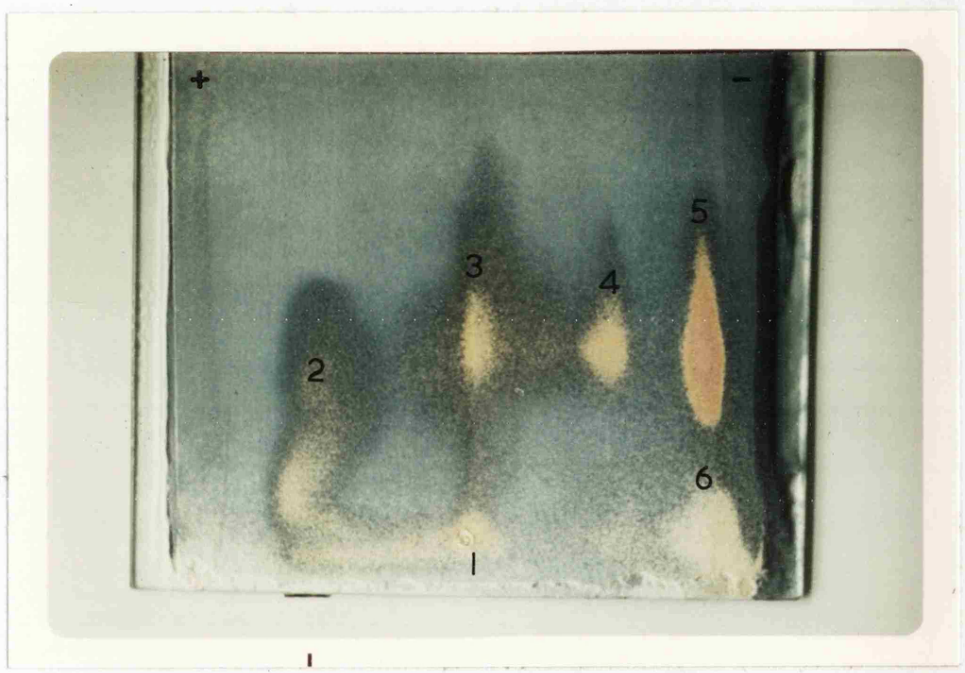
G = Enzyme.

of 100 mA, dried and visualized with chlorine/tolidene spray. The resultant chromatogram, figure 4:8, demonstrates the separation of the dipeptide from the amino acids. However, because of limitations to the quantity of reaction mixture applied to the plate and the difficulty in determining accurately the quantity of dipeptide formed relative to the reaction mixture amino acid concentrations applied to the plate, the technique was not considered as being suitable for accurate concentration determinations.

(iii) *Precipitation /Crystallization Methods*

The separation of the dipeptide as a pure crystalline solid from the other components of the reaction mixture requires that there be sufficient dipeptide synthesized during the reaction to be precipitated and purified by recrystallization. This would require a yield of at least 20 mg dipeptide; and at 1% synthesis , a reaction system consisting of 1.2 g CBZ glycine and 0.92 g phenylalanine amide in 1,150 ml buffer would be required. Since such quantities cannot be conveniently handled the alternative was to add dipeptide to the reaction mixture to raise the concentration to a convenient level to allow separation. The difficulty was to estimate the amount of dipeptide added in the amount of pure dipeptide recovered after the separation technique since, although the ratio of

Fig. 4:8. Resolution of Reaction Mixture by Combined Chromatography and Electrophoresis.



1. = Origin; Enzyme.

2. = CBZ glycine.

3. = CBZ-gly-phe-amide.

4. = ? (Enzyme?).

5. = L-phenylalanine amide.

6. = Enzyme?

dipeptide added to dipeptide synthesized remains constant, variable losses during the separation and recrystallization procedures produces variable yields of dipeptide. The problem was surmounted by using isotopically labelled reactants and either starting off the reaction with labelled dipeptide and measuring the labelled dipeptide remaining after reaction, or starting the reaction with labelled amino acid and measuring the labelled dipeptide formed. The addition of unlabelled dipeptide to facilitate the separation procedure was allowed for by calculating the difference in the specific activities of the initial reactant and final product.

The reaction system from which the dipeptide had to be separated now consisted of:-

CBZ gly	5×10^{-3}M (26 mg/25 ml)
Phe Amide	5×10^{-3}M (20 mg/25 ml)
CBZ-gly-phe-amide	1×10^{-2}M (0.4 mg + 80 mg/25 ml) (synthesized + added)
Enzyme	0.2 mg/ml

in $KCl/CaCl_2$ buffer, or Tris/HCl buffer.

A variety of liquids were tried as recrystallizing solvents for CBZ gly phe amide. Water was initially used to recrystallise the dipeptide, but occasionally oil formation resulted, especially in the presence of CBZ glycine which was

added to dilute out the contaminating radioactive amino acid. Chloroform was tried with similar problems of oil formation. The best system for the recrystallization of CBZ-gly-phe-amide was finally determined as methanol/water. The dipeptide was initially dissolved with warming in the minimum of methanol, cooled in ice, and water added dropwise until a thin white precipitate formed. On further cooling and scratching the vessel wall with a spatula, crystallization was induced.

The separation, by precipitation, of the dipeptide from the other reaction mixture components was achieved by a three stage process. After the termination of reaction a suitable volume, usually 20 ml, of reaction mixture were pipetted into 100 ml of ice cold acetone containing a weighed amount (approximately 80 mg) of dipeptide carrier. This ensured the intimate mixing of the synthesized and added dipeptides. The second stage involved taking the resulting solution down to dryness on a Buchi rotary evaporator, initially at 40°C to remove the acetone and finally at 60°C to remove the aqueous fraction. In the final stage the dried residue in the flask was redissolved in the recrystallization solvent and the dipeptide crystallized from it. In the case of water as recrystallizing solvent, the entire contents of the flask were redissolved before precipitation of the dipeptide, but in the case of methanol/water solvent, the methanol redissolved only the amino acids and dipeptide, the buffer components remaining as a solid residue.

The efficiency of recrystallization and the purity of the end product were measured by utilizing the radioactive properties of the substrates. The efficiency of recrystallization was determined by relating the number of counts in a dipeptide sample isolated from the radioactive reaction mixture, with its optical density. On first precipitating the dipeptide from the recrystallization solvent at the final stage of separation, a few crystals were removed, dissolved in 2.0 ml water with warming and the optical densities of the solution determined in 2 x 10 mm cuvettes at 223 nm and 250 nm on a Pye Unicam SP500 spectrophotometer, these wavelengths representing those points in the spectrum where the largest difference in optical density between the dipeptide and CBZ glycine existed (see Fig. 4:1). The millimolar absorbance of the dipeptide at 223 nm is 1.51, the amino acid, 0.14; and at 250 nm, the dipeptide 0.30 and CBZ glycine 0.15. An aliquot of this solution was then transferred to a liquid scintillation vial, 5.0 ml Unisolve scintillation phosphor added and the vial retained for counting on the Phillips scintillation counter. This procedure was repeated for each recrystallization of the dipeptide, the radioactivity in each sample expressed as counts per minute per ml. solution and the ratio counts per minute per ml to optical density at 223 nm and 250 nm calculated. These figures are presented in Table 4:1 and indicate that four recrystallisations are sufficient for the purification of the dipeptide sample.

Table 4:1

Crystallization stage	OD_{223}	OD_{250}	cpm/ml	Ratio cpm/OD_{223}	Ratio cpm/OD_{250}
Initial precipitation	0.280	0.069	206	736	2985
Recrystallization 1	0.300	0.075	106	353	1413
Recrystallization 2	0.328	0.077	64	195	831
Recrystallization 3	0.496	0.105	71	143	676
Recrystallization 4	0.479	0.115	67	140	583
Recrystallization 5	0.415	0.097	58	140	598

The purity of the final dipeptide, product was assessed using several techniques. The ratio of the optical densities of the dipeptide solution at 223 nm and 250 nm was one criterion to estimate product purity. The standard ratio, calculated from the absorbances in Figure 4:1 was 4.9. However experimental values from dipeptides isolated from reaction mixtures varied from 3.2 to 5.3, absorbances in the lower part of the ultraviolet region of the spectrum being significantly effected by the slightest contamination of either recrystallized material, solvent or cuvette. This method was abandoned in favour of reaction mixture blanks. Blanks were prepared by making up excess reaction mixture at the beginning of each reaction run and, before the addition of enzyme, removing an aliquot and storing it in the refrigerator during the experimental reaction. This unreacted blank was included in the separation and purificiation procedure with the dipeptide from the reaction system, all counts in this sample being due to radioactive amino acid contamination. It was found that a constant contamination of 25 to 30 counts above background remained in the dipeptide sample, which could not be easily removed, but could be corrected for in the calculations. The melting point of each dipeptide sample was also measured and compared against a standard dipeptide melting point of 125.5 - 126.5^{o}C to ensure no gross contamination of the sample occurred from a non-radioactive component.

CHAPTER 5

THE DETERMINATION OF IONIZATION CONSTANTS

5:1 INTRODUCTION

The calculation of the equilibrium constant for the peptide bond forming reaction according to equation 4:31 requires the determination of the concentrations of the unionized dipeptide product and the ionized reactant amino acids. At neutral pH CBZ glycine is fully ionized but L-phenylalanine amide is only partially ionized. In order to calculate the concentration of ionized L-phenylalanine amide the dissociation constant of the compound must be measured. Since this study involved the calculation of the standard free energy of formation at elevated temperatures, the effect of temperature on ionization constants also had to be considered.

A small change in pH can cause a large change in the percentage ionization of a base or acid, particularly if the values of pH and pKa lie close together, as they do at the point of half neutralization. The ionization process for a weak acid (HA) may be illustrated by the following equilibrium:

$$HA \rightleftharpoons H^+ + A^- \qquad 5:1$$

The ionization constant for this reaction is:

$$Ka^T = \frac{[H^+][A^-]}{[HA]} \qquad 5:2$$

where the values in brackets represent the activity of the species involved. Similarly for a weak base the ionization is:

$$BH^+ \rightleftharpoons H^+ + B \qquad 5:3$$

and

$$Ka^T = \frac{[H^+][B]}{[BH^+]} \qquad 5:4$$

At a given temperature the constants Ka^T are thermodynamic quantities and are known as the thermodynamic ionization constants. These constants are independent of concentration since all the terms are expressed as activities. The concentration ionization constant, Ka^C, is defined for acids as:

$$Ka^C = \frac{(H^+)(A^-)}{(HA)} \qquad 5:5$$

and for bases as

$$Ka^C = \frac{(H^+)(B)}{(BH^+)} \qquad 5:6$$

where the quantities of the components are expressed in stoichiometric molar concentrations. The difference between Ka^T and Ka^C constants is in the use of activities for Ka^T, which allows for the interaction between the species involved in the equilibrium. The smaller the concentration of components the less this interaction becomes until at infinite dilution $Ka^C = Ka^T$.

Ionization constants are small and inconvenient figures so they are usually expressed as their negative logarithms, pKa values.

The association between pH and pKa may be derived from equations 5:5 or 5:6. For example, taking the logarithms of equation 5:5.

$$\log Ka = \log (H^+) + \log (A^-) - \log (HA) \qquad 5:7$$

By rearrangement:

$$-\log Ka = -\log (H^+) + \log \frac{(HA)}{(A^-)} \qquad 5:8$$

Now $-\log Ka = pKa$

and $-\log (H^+) = pH$

So:

$$pKa = pH + \log \frac{(HA)}{(A^-)} \qquad 5:9A$$

or

$$pKa = pH + \log \frac{(BH^+)}{(B)} \qquad 5:9B$$

and

$$pH = pKa + \log \frac{(A^-)}{(HA)} \text{ for acids} \qquad 5:10A$$

and

$$pH = pKa + \log \frac{(B)}{(BH+)} \text{ for bases} \qquad 5:10B$$

where (A^-) or (B) can be any base and (HA) or (BH^+) its associate acid. Equation 5:10, the Henderson - Hasselbach equation, is the standard expression used for the calculation of percentage ionization in a solution of acid or base of known pH and pKa.

The effect of temperature on the pKa value must be considered. Ionization constants vary with temperature, the correlation curve

usually being a parabola with a flattened maximum. For many acids, including all carboxylic acids, the maximum lies near 20 to 25°C. Non-carboxylic acids, in general, are found to be particularly temperature sensitive increasing by up to 0.012 pKa units per degree rise in temperature (112). Nitrogenous bases are highly temperature sensitive and become weaker as the temperature increases, the effect being greater the stronger the base. Perrin (113) has investigated the effect of temperature on the pKa values of organic bases and has derived the following formula for monoacidic bases:

$$\frac{-d\ (pKa)}{dT} = \frac{pKa + 0.218\ \Delta S^{o}}{T} \qquad 5:11$$

where T is the temperature in degrees absolute and ΔS^{o} the entropy of ionization. By determining ΔS^{o} for proton loss from monocations of various organic bases Perrin converted the equation to:

$$\frac{-d\ (pKa)}{dT} = \frac{pKa - 0.9}{T} \qquad 5:12$$

However, for temperatures much removed from 25°C (i.e. 25°C ±10°C) the constant, 0.9, has a different value since ΔS^{o} is temperature dependent. Perrin was unable to derive an equation for the variation of pKa with temperature in organic acids because the reaction does not balance electrically (unlike $BH^{+} \rightleftharpoons B + H^{+}$) and because of this solvation effects varied more.

Since temperatures up to 80°C were to be used in this work it was decided to measure the pKa's of the relevant compounds,

L-phenylalanine amide and L-leucine amide, at temperatures of 25°, 40°, 60° and 80°C.

5:2 THE DETERMINATION OF IONIZATION CONSTANTS.

(a) Methods of Ionization Constant Determination.

Ionization constants may be determined several ways. *Potentiometric titration* is by far the most convenient method of determining ionization constants. By serially adding a known amount of acid to a known amount of base, or vice versa, accurately measuring the change in pH and substituting the results into equation 5:9, the pKa of a compound may be directly determined. The method requires the accurate measurement of pH using two half cells, usually glass electrodes. One is a reversible electrode whose potential changes as the hydrogen ion activity changes, the other is the reference electrode whose potential is known and remains constant throughout. The potential difference between the two electrodes in solution is a measure of the hydrogen ion activity or pH of that solution. The most commonly used electrode system for potentiometric titrations is the glass measuring electrode and the saturated calomel reference electrode. A series of *indicators* may be used instead of electrodes to measure the pH change during the titration. However, it is rather a tedious method and rarely used now.

Spectrophotmetry is the most commonly used method for the direct observation of mixtures of ionic and molecular species at known pH.

It is particularly suitable for sparingly soluble substances and also for work at high or low pH values which are beyond the range of the glass electrode. This technique may only be used with substances which absorb in ultra violet or visible light and the relevant ionic or molecular species must have different spectra. The method is related to potentiometry since spectrophotometric determinations are carried out in buffers whose pH values have been measured potentiometrically. *Proton magnetic resonance or n.m.r. spectroscopy* is useful for substances whose spectra do not change upon ionization and which are too weak as acids or bases to be measured by potentiometry. A series of solutions of known pH are prepared and the chemical shift of a non-exchanging proton near to the ionizing group is plotted against pH, the pKa being read off at the point produced by extrapolation of the two straight lines either side of the region of inflection of the curve. The accuracy of the method is limited by the relatively high concentrations necessary for n.m.r. signals. The *conductimetric* determination of ionization constants depends upon the change in conductivity that occurs when a moderately weak acid is successively diluted with water. It is not a very versatile method but, in modified form, has been found useful for very weak acids. Of the techniques available the most convenient, direct and accessible method of ionization constant determination is potentiometry. This technique was used to measure the pKa values of L-phenylalanine amide and L-leucine amide at various temperatures.

(b) The Potentiometric Measurement of the pKa Values of L-Phenylalanine Amide and L-Leucine Amide.

The apparatus used in these experiments consisted of a Metrohm type EA 109H high temperature glass electrode (U.K. distributor: Shandon Southern Instruments Ltd.) and a Metrohm type EA 425 Ag/AgCl reference electrode. Both electrodes were new at the start of the measurements and were pre-soaked in distilled water for 24 hours before use. In between measurements they were stored in distilled water. The pH meter was a Metrohm type E512 transistorized pH meter which could be calibrated to 0.005 pH units. The pH determinations were carried out using an Agla micrometer syringe (Burroughs, Wellcome and Co.) to deliver titrant (accuracy ±0.00005 ml) to the solution under investigation in a 20 ml capacity Metrohm water-jacketed titration vessel. Water was circulated through the vessel from a thermostatically controlled water bath (Grants Instruments (Cambridge) Ltd) by means of a centrifugal water pump (Grants Ltd) at approximately 12 litres/min. The liquid in the titration vessel was stirred initially by means of a magnetic stirrer until temperature equilibration, but during the titration run stirring was accomplished by a slow stream of nitrogen bubbles. The gas, high purity nitrogen (Air Products Ltd) was saturated with water before being introduced under the surface of the solution to be titrated. The pH meter and electrodes were calibrated for each experiment at each temperature with two standard buffer solutions:

Buffer A: Potassium dihydrogen orthophosphate,

$m = 0.025$ mol kg^{-1}

Disodium hydrogen orthophosphate,

$m = 0.025$ mol kg^{-1}

pH = 6.865 at 25°C

Buffer B: Potassium dihydrogen orthophosphate,

$m = 0.008695$ mol kg^{-1}

Disodium hydrogen orthophosphate,

$m = 0.03043$ mol kg^{-1}

pH = 7.413 at 25°C.

Data for the change in pH of the above buffers with temperature was obtained from the Handbook of Biochemistry (55). The relevant information is reproduced in Table 5:1

Table 5:1

Temperature °C	pH, buffer A	pH, buffer B
25	6.865	7.413
40	6.838	7.386
60	6.836	-
80	6.859	-

Occasionally the condition of the electrodes was checked by calibration of the pH assembly with buffer A at 25°C and then measurement of potassium hydrogen phthalate buffer, m = 0.05 mol kg^{-1}, pH 4.008 at 25°C. Any variation greater than 0.03 pH units, the electrodes were cleaned or replaced.

Phenylalanine amide. A 0.01 M solution of L-phenylalanine amide in CO_2 free double distilled water was prepared and 25.0 ml pipetted into the water-jacketed titration vessel and allowed to warm to the required temperature. The titrant, 0.5 M HCl in CO_2 free double distilled water was prepared from a concentrated volumetric solution (B.D.H. Ltd.) and the Agla syringe filled. The glass electrode was standardized with buffers A and B at the required temperature, the electrodes immersed in the phenylalanine amide solution and the nitrogen stream started. After allowing several minutes for equilibration, the pH was read. The titrant was added in ten equal portions, each a tenth of an equivalent, and the pH recorded as soon as equilibration had been reached after each addition . Upon completion the electrodes were washed with water and the calibration rechecked in buffer A. If the variation was greater than 0.03 pH units the titration was repeated. Calculation of the results by substitution in equation 5:9B is relatively simple. If the pH remains between 4 and 10 when an 0.01 M solution is titrated with a solution at least ten times as concentrated, no corrections for activities or dilution by the titrant are necessary (112).

Table 5:2 represents a typical pKa determination.

Table 5:2

Substance: L-Phenylalanine amide. Temperature 25°C

Concentration: 0.01 M at half neutralization: Titrant: 0.5 M HCl

1	2	3	4	5	6	7	8
Titrant (ml)	pH	Stoicheiometric concentrations (BH^+)	(B)	$\frac{(BH^+)}{(B)}$	$\log\frac{(BH^+)}{(B)}$	pKa =pH +col 6	Antilog pKa
0	(8.9)	0	0.01	-	-	-	-
0.05	8.185	0.001	0.009	1/9	-0.95	7.235	5290
0.10	7.860	0.002	0.008	2/8	-0.60	7.260	5321
0.15	7.630	0.003	0.007	3/7	-0.37	7.260	5321
0.20	7.440	0.004	0.006	4/6	-0.18	7.260	5321
0.25	7.260	0.005	0.005	5/5	0	7.260	5321
0.30	7.090	0.006	0.004	6/4	+0.18	7.270	5333
0.35	6.895	0.007	0.003	7/3	+0.37	7.265	5327
0.40	6.660	0.008	0.002	8/2	+0.60	7.260	5321
0.45	6.285	0.009	0.001	9/1	+0.95	7.255	5319
0.50	(4.05)	0.01	0	-	-	-	-

Average = 5319

Literature value = 7.22 pKa = 7.258 + 0.023

Table 5:3 (a)

L-Phenylalanine Amide at 40°C

Titrant (ml)	pH	log $\frac{(BH^+)}{(B)}$	pKa	Antilog pKa
0	(>8.4)	-	-	-
0.05	7.820	-0.95	6.870	4864
0.10	7.485	-0.60	6.885	4881
0.15	7.300	-0.41	6.890	4887
0.20	7.105	-0.21	6.895	4893
0.25	6.925	-0.03	6.895	4893
0.30	6.755	+0.14	6.895	4893
0.35	6.565	+0.33	6.895	4893
0.40	6.360	+0.55	6.910	4909
0.45	6.020	+0.86	6.880	4875
0.50	(<5.6)	-	-	-

pKa = 6.890 ± 0.020 Calculated value = 6.917 (equation 5:12)

Table 5:3(b)

L-Phenylalanine Amide at 40°C

Titrant (ml)	pH	Log $\frac{(BH^+)}{(B)}$	pKa	Antilog pKa
0	(>8.4)	-	-	-
0.05	7.785	-0.95	6.835	4825
0.10	7.445	-0.60	6.845	4837
0.15	7.240	-0.37	6.870	4864
0.20	7.055	-0.18	6.875	4870
0.25	6.880	0	6.880	4875
0.30	6.710	+0.18	6.890	4887
0.35	6.520	+0.37	6.890	4887
0.40	6.285	+0.60	6.885	4881
0.45	5.930	+0.95	6.880	4875
0.50	(<5.6)	-	-	-

pKa = 6.872 ± 0.037 Calculated value = 6.917 (equation 5:12)

Table 5:4

L-Phenylalanine Amide at 60°C

Titrant (ml)	pH	$\log\frac{(BH^+)}{(B)}$	pKa	Antilog pKa
0	8.295	-	-	-
0.05	7.365	-0.95	6.415	4380
0.10	7.040	-0.60	6.440	4406
0.15	6.820	-0.37	6.450	4416
0.20	6.635	-0.18	6.455	4421
0.25	6.465	0	6.465	4431
0.30	6.285	+0.18	6.465	4431
0.35	6.100	+0.37	6.470	4436
0.40	5.865	+0.60	6.465	4431
0.45	(5.45)	+0.95	-	-
0.50	(4.35)	-	-	-

pKa = 6.441 ± 0.029

Table 5:5

L-Phenylalanine Amide at 80°C

Titrant (ml)	pH	$\log\frac{(BH^+)}{(B)}$	pka	Antilog pKa
0	(8.20)	-	-	-
0.05	7.065	-0.95	6.115	4088
0.10	6.730	-0.60	6.130	4102
0.15	6.495	-0.37	6.125	4098
0.20	6.300	-0.18	6.120	4093
0.25	6.125	0	6.125	4098
0.30	5.957	+0.18	6.137	4109
0.35	5.765	+0.37	6.135	4107
0.40	(5.45)	+0.60	-	-
0.45	(5.10)	+0.95	-	-
0.50	-	-	-	-

pKa = 6.127 ± 0.012

The scatter of the quoted pKa value is calculated by taking the antilog. of each pKa reading, averaging them, quoting the logarithm of this average as the pKa and taking the largest deviation between this value and any other value in the set of readings as its scatter. Any scatter exceeding 0.06, the titration was repeated.

This procedure was repeated for each temperature. The results are shown in tables 5:3 to 5:5 and summarised in table 5:6.

Table 5:6

The Effect of Temperature on the Ionization Constant of L-Phenylalanine Amide

Temperature (^{o}C)	pKa
25	7.26 ± 0.02
40 (a)	6.89 ± 0.02
40 (b)	6.87 ± 0.04
60	6.44 ± 0.03
80	6.13 ± 0.01

Leucine Amide. Leucine amide is only supplied as its salt L-leucine amide hydrochloride. A 0.01 M solution of L-leucine amide HCl was prepared in CO_2 free double distilled water and 25.0 ml used for each pKa determination. The titrant

was 0.5 M potassium hydroxide solution, prepared from a concentrated volumetric solution (BDH Ltd) and stored in a stoppered bottle protected by a soda-lime tube. Potassium hydroxide was used in preference to sodium hydroxide because the glass electrode is able to tolerate a higher ratio of potassium to hydrogen ions than sodium to hydrogen ions before the accuracy of the electrode response is effected. The pKa determinations were carried out in the same manner as for phenylalanine amide, table 5:7 showing a typical L-leucine amide pKa determination.

The pKa values for temperatures 40, 60 and 80^{o}C are shown in tables 5:8 to 5:10 and summarized in table 5:11.

The amount of ionized L-phenylalanine amide or L-leucine amide present in the total concentration of the particular amino acid in the reaction mixture at equilibrium, may be calculated from equation 5:10 by substitution of the pH of the reaction mixture and the derived pKa value into the equation and solving for (A^{-}). The value is expressed as a proportion of the total concentration of amino acid, for example: L-phenylalanine amide at 25^{o}C and at pH 8.0, y = 0.154Y, where y is the concentration of ionized phenylalanine amide and Y is the total concentration of that amino acid.

Table 5:7

Substance : L-Leucine amide HCl Temperature 25°C

Concentration: 0.01M at half neutralization. Titrant 0.5M KOH

1	2	3	4	5	6	7	8
Titrant (ml)	pH	Stoichiometric concentrations (HA)	(A^-)	$\frac{(HA)}{(A^-)}$	$\log\frac{(HA)}{(A^-)}$	pKa = pH + col 6	Antilog pKa
0	(4.95)	0.01	0	-	-	-	-
0.05	6.855	0.009	0.001	9/1	+0.95	7.805	6033
0.10	7.195	0.008	0.002	8/2	+0.60	7.795	6019
0.15	7.420	0.007	0.003	7/3	+0.37	7.790	6012
0.20	7.600	0.006	0.004	6/4	+0.18	7.760	5970
0.25	7.775	0.005	0.005	5/5	0	7.775	5991
0.30	7.950	0.004	0.006	4/6	-0.18	7.770	5984
0.35	8.140	0.003	0.007	3/7	-0.37	7.770	5984
0.40	8.370	0.002	0.008	2/8	-0.60	7.770	5984
0.45	(8.50)	0.001	0.009	1/9	-0.95	-	-
0.50	(9.28)	0	0.01	-	-	-	-

Average = 5997

Literature value = 7.80

pKa = 7.77 ± 0.026

Table 5:8

L-Leucine Amide at 40°C

Titrant (ml)	pH	$\log\frac{(HA)}{(A^-)}$	pKa	Antilog pKa
0	(<5.6)	-	-	-
0.05	6.450	+0.95	7.400	5495
0.10	6.775	+0.60	7.375	5464
0.15	7.000	+0.37	7.370	5458
0.20	7.185	+0.18	7.365	5451
0.25	7.360	0	7.360	5445
0.30	7.525	-0.18	7.345	5426
0.35	7.715	-0.37	7.345	5426
0.40	7.930	-0.60	7.330	5408
0.45	8.240	-0.95	7.290	5358
0.50	(>8.4)	-	-	-

pKa = 7.350 ± 0.06

Calculated value = 7.47
(Equation 5:12)

Table 5:9

L-Leucine Amide at 60°C

Titrant (ml)	pH	$\log\frac{(HA)}{(A^-)}$	pKa	Antilog pKa
0	(4.50)	-	-	-
0.05	6.040	+0.95	6.990	5000
0.10	6.385	+0.60	6.985	4995
0.15	6.610	+0.37	6.980	4989
0.20	6.800	+0.18	6.980	4989
0.25	6.975	0	6.975	4983
0.30	7.155	-0.18	6.975	4983
0.35	7.350	-0.37	6.980	4989
0.40	7.595	-0.60	6.995	5006
0.45	7.955	-0.95	7.005	5018
0.50	(8.81)	-	-	-

pKa = 6.985 ± 0.02

Table 5:10

L - Leucine Amide at 80°C

Titrant (ml)	pH	$\log\frac{(HA)}{(A^-)}$	pKa	Antilog pKa
0	(4.38)	-	-	-
0.05	5.720	+0.95	6.670	4645
0.10	6.042	+0.60	6.642	4615
0.15	6.270	+0.37	6.640	4613
0.20	6.455	+0.18	6.635	4608
0.25	6.622	0	6.622	4595
0.30	6.800	-0.18	6.620	4592
0.35	7.005	-0.37	6.635	4608
0.40	7.225	-0.60	6.625	4597
0.45	7.560	-0.95	6.610	4581
0.50	8.382	-	-	-

pKa = 6.634 ± 0.036

Table 5:11

The Effect of Temperature on the Ionization Constant of L-Leucine Amide

Temperature °C	pKa
25	7.78 ± 0.03
40	7.35 ± 0.06
60	6.98 ± 0.02
80	6.63 ± 0.04

CHAPTER 6

THE DETERMINATION OF THE FREE ENERGIES AND HEATS OF PEPTIDE BOND FORMATION

6:1 INTRODUCTION

In a reaction system there are several factors which affect the position and attainment of equilibrium and thus the calculated equilibrium constant and free energy values. These factors are reactant and product effects, including amino acid and dipeptide concentrations, activities, optical purities and degrees of ionization; enzyme effects; the effect of pH and reaction conditions and product purity. All these factors had to be taken into consideration in order to design the most appropriate experimental reaction conditions.

6:2 REACTANT AND PRODUCT EFFECTS

(a) Amino Acids. The concentration of amino acids may affect the amount of dipeptide synthesized in several ways.

The relative amount of dipeptide synthesized in a reaction system decreases as the initial concentration of the amino acids is decreased. This may be best illustrated by reference to Table 6:1 adapted from Borsook (2).

As the initial amino acid concentration decreases by a factor of ten, the percentage of dipeptide synthesized also falls by a factor of ten, but the actual concentration of dipeptide formed falls by a factor of one hundred. This relationship is common

Table 6:1

Initial Amino Acid Concentration (M)	Equilibrium Constant	Dipeptide Concentration at Equilibrium (M)	% Dipeptide Synthesized at Equilibrium
0.1	0.56	5.6×10^{-3}	5.6
0.01	0.56	5.6×10^{-5}	0.56
0.001	0.56	5.6×10^{-7}	0.056
0.0001	0.56	5.6×10^{-9}	0.0056

to all reactions where two molecules condense to form one. Thus a choice has to be made, dependent upon the equilibrium constant for the reaction, (the smaller the equilibrium constant the less favoured dipeptide synthesis becomes), between a relatively high amino acid concentration and its related dipeptide concentration, which makes the task of dipeptide concentration determination easier but requires the use of larger quantities of labelled amino acid, and a relatively low amino acid concentration with its subsequent very small amount of synthesized dipeptide.

Related to the choice of amino acid concentration is a second factor which independantly affects the amount of dipeptide synthesized. This factor is activity. The activity (A) of a solute may be defined as the concentration of solution (C) multiplied by a correction factor, the activity coefficient (Ψ), to give the active mass of solute.

ie. $$A = \Psi \times C$$

A study of values of the equilibrium constant, K, over a range of amino acid concentrations would show that K calculated from the concentrations of the components is not constant. This is because the amino acid solution does not behave, from a thermodynamic point of view, as an ideal solution since ideal behaviour is only found when there are no forces of attraction between the solute molecules and when they occupy a neglibible volume compared with the total

volume. The greatest variation occurs in cases of equilibrium involving ions, since interionic attraction causes the largest deviations from ideal behaviour. By substituting activities for concentrations in the determination of the equilibrium constant deviations from ideality are compensated for. The calculation of Ψ is unfortunately arduous and lengthy. However, because the deviation from ideal behaviour varies with concentration, the activity coefficient also does so. In very dilute solutions Ψ approaches unity making A approach C . Thus in very dilute solutions amino acids may be regarded as behaving ideally. The definition of 'very dilute solution' varies depending upon the electrolyte (or non-electrolyte) considered. In the case of protected amino acids and dipeptides, concentrations of 0.2M and less were considered as approaching ideality, based on activity figures collected by Hutchens (58).

Dipeptide synthesis may also be influenced by the optical form of the reactant amino acids. Glycine, in the form of CBZ glycine, does not have optical isomers, but phenylalanine does. Phenylalanine amide may exist as either the D- or L- isomer. The enzyme catalysing the peptide bond forming reaction, thermolysin, requires the L-configuration at the active site (79). In order to establish the stability of L-phenylalanine to racemization under reaction conditions the following investigation was carried out: Solutions of L-phenylalanine amide in water, 1 M HCl and 0.01 M

Tris/HCl buffer pH 8.0 with 0.01M $CaCl_2$, were maintained at temperatures of 25°C and 60°C for various lengths of time. The specific rotation of the solutions were measured in a 1.0 dm. cell on a Bellingham and Stanley Model A polarimeter with half shadow polariser using a sodium lamp, before and after heating. The results are presented in Table 6:2

Table 6:2

Solution	Time Heated	$[\alpha]_D^{24°C}$	$[\alpha]_D^{60°C}$
L-phenylalanine amide in water	0	+14.3°, +14.3°	
	30 min	+14.9°, +14.9°	
	1 hr	+14.9°, +14.9°	+14.9°, +14.9°
	20 hr		+ 16.4°, +16.4°
L-phenylalanine amide in 1M HCl	0	+16.4°, +16.4°	
	1½ hr	+16.9°, +16.4°	
L-phenylalanine amide in Tris/HCl buffer	0	+13.5°, +14.3°	
	4 hr		+16.1°, +15.0°
			+15.6°

The results indicate that L-phenylalanine amide is stable at both laboratory and elevated temperature in aqueous, acid, or alkaline buffered solution. The poor solubility of the dipeptide in aqueous solution prevented the determination of its specific rotation under reaction system conditions.

The final amino acid factor which affects the determination of the equilibrium constant is the correction for ionization. The determination of this factor has been described in detail in Chapter 5. Once the reaction system had been established this correction was applied to the phenylalanine amide and leucine amide concentrations in all determinations of the equilibrium constant.

(b) Dipeptide. The concentration of dipeptide synthesized in the reaction systems was of major importance, because of the limited solubility of the compound in aqueous solution. If the solubility of the dipeptide is less than the equilibrium concentration predicted from the equilibrium constant, the percentage synthesis will be significantly increased by the dipeptide coming out of solution until the reactants are so reduced in concentration that equilibrium is reached in saturated dipeptide solution. To ensure that the equilibrium observed in the reaction mixture was not influenced by dipeptide insolubility, the solubility of CBZ-gly-phe-amide was accurately measured.

A concentrated solution of CBZ-gly-phe-amide was prepared in ethanol, and various aliquots corresponding to a series of dipeptide concentrations at constant volume, were pipetted into ampoules and taken down to dryness. 5.0 ml of 0.01M Tris/HCl buffer, pH 8.0 with 0.01 M $CaCl_2$ containing 5×10^{-3}M CBZ glycine and phenylalanine amide was pipetted into each ampoule, the contents mixed and autoclaved at 15 lbs/sq. in. for 15 minutes. The ampoules were then

sealed with a flame. This ensured no bacterial contamination of the dipeptide aliquots. Initial optical density readings at 216nm were made on diluted aliquots of the solutions remaining using a Pye Unicam SP 500 Series II spectrophotometer. The ampoules were placed in an end-over-end shaker in a water bath at 25°C for 72 hours before being opened and the precipitated material centrifuged down at 4,000 rpm for 15 minutes in a bench centrifuge. Aliquots of the solutions were suitably diluted and the optical densities read at 216 nm. The figures indicated that the limit of solubility of the dipeptide appeared to be between 1×10^{-5} M and 1×10^{-4}M and that it was stable to autoclaving and therefore to elevated temperatures in Tris buffered solution. More accurate determination of the dipeptide solubility in this system was prevented by the need to dilute the solutions considerably before measurement due to optical density contributions by the amino acids. To overcome this problem a series of dipeptide concentrations in 0.03M KCl, (equivalent to 0.01M Tris/HCl + 0.01M $CaCl_2$ + 0.01M amino acids), which does not absorb in the U.V. region at 216 nm, were prepared. These solutions were treated similarly to the Tris/HCl solutions and the resulting final optical densities plotted against dipeptide concentration. The resultant curve is presented in Figure 6:1.

The maximum solubility of CBZ-gly-phe-amide in 0.03M ionic solution at 25°C is 1×10^{-4}M.

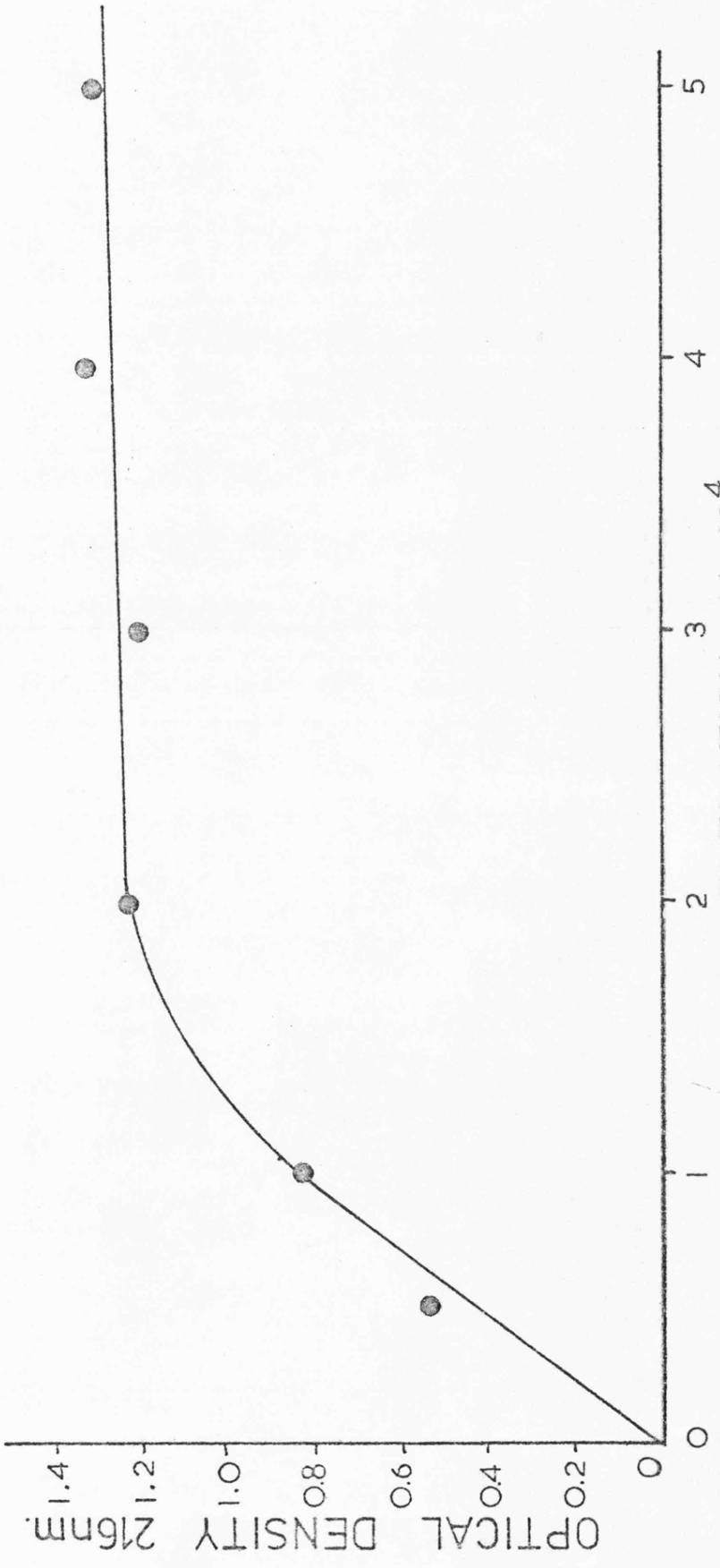


Fig 6:1. Solubility Curve of CBZ-gly-phe-amide in 0.03M KCl.

A second factor which may affect the amount of dipeptide synthesized is the stability of the peptide bond under reaction system conditions. This problem was answered during the dipeptide solubility determinations where it was demonstrated that the dipeptide was stable in solution at temperatures of 100°C at 15 lbs/sq. in. pressure for 15 minutes.

Taking into consideration all the factors mentioned above an assessment of the possible reaction mixture concentrations was made. On the assumption that the amount of dipeptide synthesized would be 1% of the initial amino acid concentrations and knowing the maximum solubility of the dipeptide at 25°C was 1×10^{-4} M, the initial concentrations of the two reactant amino acids must not exceed 1×10^{-2} M. Since ionization corrections applied to phenylalanine amide and leucine amide to bring the corrected concentrations down to 1×10^{-2} M would mean actual initial concentrations in excess of this figure, amino acid concentrations of 5×10^{-3} M were chosen as the standard reaction mixture initial concentration. For the reverse hydrolysis reaction the maximum dipeptide concentration of 1×10^{-4} M was used.

6:3 Enzyme Effects

Based on the assumption that the enzyme acts as a perfect catalyst, (See chapter 2), the concentration of thermolysin should have no effect on the equilibrium constant. In order to minimise the chance of enzyme interference, low concentrations of enzyme were used in reaction systems where possible, but a balance between

the concentration of enzyme and the time of reaction had to be made. Two concentrations of enzyme were used, initially 3×10^{-6} M and finally 2×10^{-8} M. Rate data, based on an initial rate value of 3.5 μm dipeptide hydrolysed/min/mg enzyme at 60°C (see Chapter 2), gave times of 15 minutes and 34 hours for the complete hydrolysis of 125 μm of substrate (equivalent to 25 ml of solution at a concentration of 5×10^{-3} M) at the two enzyme concentrations. As the amount of dipeptide synthesized was expected to be around 1% of reactant concentration, the above enzyme reaction times were corrected for the complete hydrolysis of 1.25 μm of substrate. The values were also corrected for enzyme operation at 25°C, an arbitrary initial rate value of 1 μm hydrolysed/min/mg enzyme at 25°C being used. Complete hydrolysis of 1.25 μm of dipeptide at 25°C was calculated as being achieved in 0.5 minutes and $1\frac{1}{4}$ hours at the two enzyme concentrations. However, to ensure that sufficient time had elapsed for a true equilibrium to be established between amino acid reactants and dipeptide product, these times were increased ten fold so that at an enzyme concentration of 3×10^{-6} M equilibrium would be established within 5 minutes and at an enzyme concentration of 2×10^{-8} M equilibrium would be reached within 12 hours. In practice most reactions were carried out for either 1 to 2 hours or overnight, 16 to 20 hours. The reverse dipeptide hydrolysis reaction was carried out at an enzyme concentration of 2×10^{-8} M. Equilibrium was calculated as being attained at about 20 hours reaction time.

6:4 THE EFFECT OF pH

It has been demonstrated that the calculated free energy data for a specific reaction varies with the formulation of that reaction (Chapter 4). The various formulations of the equations and the subsequent free energy calculations differ due to the differing ionization states of the reactant and product molecules expressed in the equation. Since the degree of ionization of a molecular species varies with pH, the absolute amount of dipeptide synthesized must also vary with pH. The variation in the amount of dipeptide synthesized with pH may be best expressed in terms of the total degree of synthesis, which is the concentration of dipeptide formed in all its ionic forms from a given total concentration of amino acids in all their ionic forms. If the total concentration of a given ionic species in all its forms with its ionization constant and the pH of the system is inserted into the mass action equilibrium expression with similar values for the other components of the system, by algebraic rearrangement an expression containing the degree of total synthesis may be obtained. In the case of CBZ-gly-phe-amide the effect of pH on the degree of synthesis may be derived as follows:

$$\begin{array}{lcl} \text{CBZ gly}^- + \text{phe amide}^+ & \rightleftharpoons & \text{CBZ-gly-phe-amide} + H_2O \\ (CG^-) + (PA^+) & & (CGPA) + H_2O \end{array} \qquad 6:1$$

$$K = \frac{(CGPA)}{(CG^-)\ (PA^+)} \qquad 6:2$$

Taking CBZ gly:

$$(CG) \xrightleftharpoons{k'} (CG^-) + (H^+) \qquad 6:3$$

and

$$\Sigma(CG) = (CG) + (CG^-) \qquad 6:4$$

$$k' = \frac{(CG^-)\ (H^+)}{(CG)} \qquad 6:5$$

Rearranging and substituting 6:4 into 6:5

$$k' = \frac{(CG^-)(H^+)}{\Sigma(CG)-(CG^-)} \qquad 6:6$$

$$\Sigma(CG)k' - (CG^-)k' = (CG^-)(H^+) \qquad 6:7$$

$$\Sigma(CG)k' = (CG^-)(H^+) + (CG^-)k' \qquad 6:8$$

$$= (CG^-)\left[\ (H^+) + k'\ \right] \qquad 6:9$$

So

$$(CG^-) = \frac{\Sigma(CG)\ k'}{(H^+)+k'} \qquad 6:10$$

and similarly

$$(PA^+) = \frac{\Sigma\ (PA)(H^+)}{(H^+)\ +k''} \qquad 6:11$$

So

$$\Sigma\ (CG) = \frac{(CG^-)(H^+) + k'}{k'} \qquad 6:12$$

and

$$\Sigma\ (PA) = \frac{(PA^+)(H^+) + k''}{(H^+)}$$

Substituting equations 6:12 into equation 6:2

$$\frac{\Sigma(CGPA)}{\Sigma(CG)\Sigma(PA)} = \frac{(CGPA)\quad k'\quad (H^+)}{(CG^-)(H^+)+k'\ (PA^+)(H^+)+k''} \qquad 6:13$$

that is:

$$\frac{\Sigma(CGPA)}{\Sigma(CG)\Sigma(PA)} = K\ \frac{k'\ (H^+)}{\left[(H^+) + k'\right]\left[(H^+)+k''\right]} \qquad 6:14$$

The degree of synthesis is equal to the equilibrium constant, K, multiplied by a term containing only ionization constants and the hydrogen ion concentration. The ionization constants k' and k'' are calculated from the pKa values previously determined (Chapter 5) and literature values. At 25°C the pKa value of CBZ glycine is 3.67, so $k' = 2.14 \times 10^{-4}$, and the pKa value of phenylalanine amide is 7.22, so $k'' = 6.03 \times 10^{-8}$. Substituting these values into equation 6:14 at pH values from 3 to 9 produced the data presented in table 6:3.

From these data the maximum synthesis of CBZ-gly-phe-amide lies between pH 5.0 and 6.0 and decreases on both sides of this region. The difference in the absolute amount of dipeptide synthesized depends upon the value of the equilibrium constant. If the constant is very low there will be very little difference in dipeptide yields between pH 5.5 and pH 8.0, however, the greater

Table 6:3

pH	Ionization term
3.0	0.18
4.0	0.68
5.0	0.95
5.5	0.97
6.0	0.94
7.0	0.62
7.2	0.50
8.0	0.14
9.0	0.02

the value of K above unity the greater the absolute amount of synthesis. Since enzyme activity is optimal between pH 6.0 and pH 8.0 (78) the maintenance of the pH of the reaction system within these limits was important, especially at elevated temperatures. The stability of 0.01 M Tris/HCl buffer, pH 7.2 with 0.01 M $CaCl_2$ to elevated temperatures was investigated. A reaction mixture solution comprising of CBZ glycine (5×10^{-3} M), phenylalanine amide (5×10^{-3} M ionized phenylalanine amide) and thermolysin (2×10^{-8} M) in Tris/HCl buffer, pH 7.2 was placed in the reaction vessel, electrodes inserted into the solution and the temperature raised in stages from 25°C to 40°C, 60°C and 80°C. At each

stage additional phenylalanine amide was added to maintain the ionized concentration at 5×10^{-3}M. The results are presented in table 6:4 and show that the pH decreases as the temperature rises.

Table 6:4

Temperature ^{o}C	pH
25	7.20
40	7.10
60	6.95
80	6.90

This pH change with temperature was checked during experimental runs and was found to be constant. The phenylalanine amide and leucine amide ionization correction factors were recalculated at these new pH values and used to correct the amino acid concentration figures in the equilibrium expression.

6:5 REACTION CONDITIONS AND PRODUCT PURITY ASSESSMENT

There are several factors of the reaction system which must be controlled or compensated for so that their effect on the final product yield may be assessed.

Control of the temperature of the reaction mixture was essential since the primary object of the work was to assess the contribution to peptide bond formation of temperature. The reaction was carried out in a water-jacketed reaction vessel, the water being pumped through the vessel at a rate of 12 litres/minute from a thermostatically controlled water bath (Grant Instruments (Cambridge) Limited), additional water temperature control being provided from a Churchill Chiller Thermo circulator for temperatures up to 60°C. The temperature of the reaction mixture was monitored with a thermometer reading to 0.25°C and the water bath temperature with a maximum and minimum thermometer. Temperature fluctuation was less than ± 1°C over a 16 hour period.

The time of reaction was critical since the reaction had to be left for a sufficient period for equilibrium to be attained. As detailed in the section under enzyme effects (6:3), the length of reaction time was estimated from enzyme rate data, the period of reaction being considerably longer than the estimated equilibrium attainment times. An indication that equilibrium had been reached was obtained by removing aliquots of reaction mixture at two time intervals one hour apart and taking them through the dipeptide purification procedure. Similar results for the amount of dipeptide synthesized suggested that equilibrium had been reached within the times under investigation. Evidence for the attainment of equilibrium may be obtained by comparing the equilibrium constants derived from reactions carried out in both directions. If the constants calculated from the synthetic and hydrolytic reactions are the same, the reaction

may be assumed to be at equilibrium.

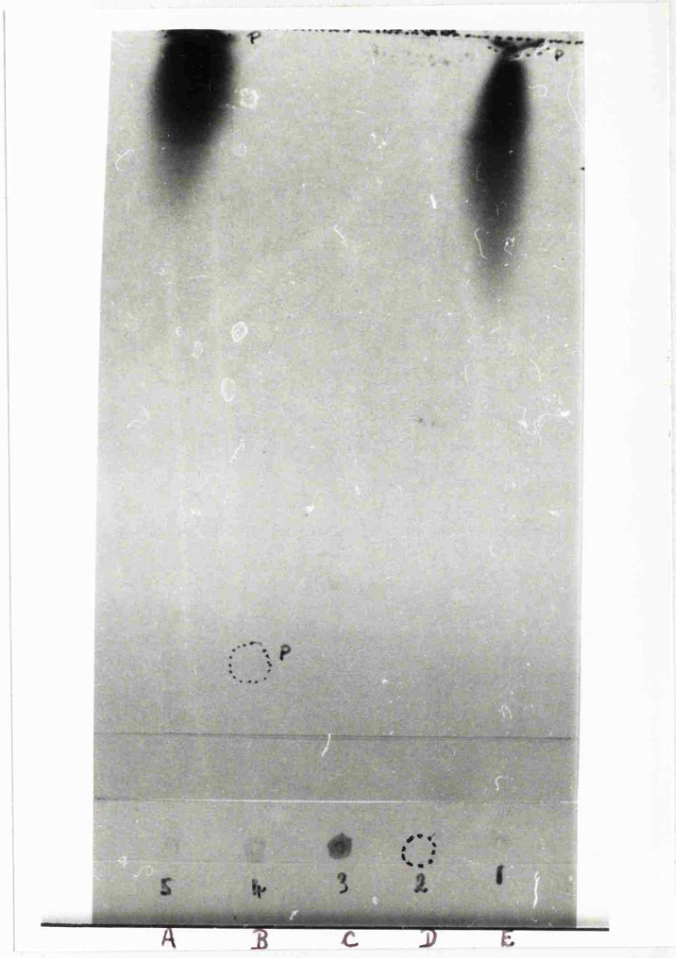
Water loss from the reaction mixture through evaporation during reaction, especially at elevated temperatures had to be controlled and measured since solvent loss affects the calculated final concentrations of reactants and products. Evaporation losses were reducedby using the reaction vessel's penton top with sealing gasket, but a significant volume of water condensed inside the top and on the vessel sides. This loss was measured by accurately pipetting the required reaction mixture volume (Vi), containing labelled reactant, into the vessel, adding the enzyme, mixing and immediately removing 1.0 ml of the solution for radioactive counting (Ci). After allowing the system to react for the required length of time a second 1.0 ml sample was removed for counting (Cf). By substitution of the initial volume and initial and final counts into equation 6:15, the final volume (Vf), of the reaction mixture could be accurately calculated.

$$Vf = \frac{Ci \times Vi}{Cf} \qquad 6:15$$

The careful assessment of product purity and an accurate measurement of final equilibrium concentrations by radioactive counting were essential for significant equilibrium constant and free energy determinations. The purity of the final dipeptide product was assessed using several techniques. During preliminary experiments the melting point of the product was always carefully

measured to ensure no gross contamination of the material. Optical density ratios and count/optical density ratios were also used initially to assess purity by removing a few crystals of product after each recrystallization, dissolving them in 2.0 ml water and measuring their optical densities at 223 nm and 250 nm on a Pye Unicam SP 500 spectrophotometer. The product was assessed as pure by either comparing the ratios O.D. 223/O.D. 250 with the same ratio of a pure dipeptide solution or by counting the solutions and expressing the result as a counts per minute/optical density relationship and aseessing the product as pure when the relationship remained constant. Further evidence of product purity was obtained by running two samples of dipeptide isolated from reaction mixtures on a thin-layer electrophoresis plate in water/acetic acid/pyridine, 970/9.8/20, v/v/v, pH 5.4 at 100 mA for 2 hours. The electrophoretogram was dried and autoradiographed with Ilford Industrial G X-ray film for 48 hours. The resultant plate is illustrated in figure 6:2. Unfortuantely these techniques only provide a relative assessment of purity. Very small quantities (of the order of 0.01% or less) of labelled amino acid contaminating the dipeptide product are undetectable by these methods. Direct indications as to the purity of the recrystallized dipeptide product were demonstarated when control experiments, in which no enzyme was added to the reaction mixture, were performed. It was found that there was a constant level of labelled amino acid contamination in the four times recrystallized dipeptide product. The level of contamination was related to the amount of unlabelled amino acid added to dilute

Figure 6:2. Autoradiogram of CBZ-gly-phe-amide.



A & E = ^{14}C CBZ glycine.

B & C = ^{14}C CBZ-gly-phe-amide sample.

D = CBZ-gly-phe-amide reference.

out contaminating labelled material during the first recrystallization of the isolated dipeptide product and also the original concentration of labelled amino acid. Maintaining the amount of unlabelled amino acid added at 50 mg per dipeptide sample, after four recrystallizations the dipeptide product contained 25 c.p.m. above background per 0.5 ml sample counted, (total volume 2.0 ml; dipeptide yield 50 - 60 mg), at initial amino acid concentrations of 5×10^{-3}M and 13 c.p.m. above background per 0.5 ml sample at initial amino acid concentrations of 2.5×10^{-3} M. This blank correction was used in the calculation of several sets of results. Subsequently for each experiment additional reaction mixture was prepared and an aliquot removed into dipeptide carrier solution before enzyme addition, stored until the remaining mixture had been reacted and then it was treated, with reaction mixture samples, to the dipeptide purification procedure. This type of internal blank determination was employed for the majority of the synthesis experiments. The blanks for the dipeptide hydrolysis reactions were obtained by electrophoresis of the dipeptide product. The purified product was dissolved in 1.5 ml acetone 2 x 0.5 ml samples were counted for specific activity determination and the remaining 0.5 ml was applied to one end of a 10 x 20 cm. 0.2 mm thick silica gel G.F. thin layer plate. A reference spot of CBZ glycine was applied at one edge of the plate before it was placed in a Shandon thin layer electrophoresis tank, allowed to soak with water/acetic acid/ pyridine, 980/9.5/10, v/v/v, buffer, pH 5.4 and run for 30 minutes at approximately 80 mA (1000 volts). The electrophoresis plate

was then dried, the silica protected with a glass sheet and the reference spot developed with chlorine-tolidene spray. The electrophoretogram was divided into squares enclosing the dipeptide (origin), CBZ glycine and blank areas and the silica scraped off into vials and counted in 5.0 ml scintillation fluid and 1.0 ml water on the Phillips automatic scintillation counter. The blank was taken as the number of counts not in the origin dipeptide square expressed as a proportion of the total number of counts on the plate.

The accuracy of the radioactive counting of the dipeptide product for final equilibrium concentration determinations was governed by several factors. To ensure the greatest number of counts possible in the isolated dipeptide, the specific activity of the reactant amino acid or dipeptide was the highest possible relative to the efficiency of the procedures of protected amino acid and dipeptide synthesis, (see Chapter 3). The greater the number of counts in the isolated dipeptide the less the radioactive counting error. The number of counts in the final dipeptide product, besides being related to the specific activity of the original labelled reactant and the actual amount of dipeptide synthesized, depends upon the amount of unlabelled dipeptide added at the end of the reaction as carrier through the purification procedure. It was found that precipitation of the dipeptide from solution and recrystallization of that product in amounts less than 50 mg created difficulties in the subsequent precipitation of the compound. The product also tended to contain greater amino acid contamination when precipitated

in small quantities. Since four recrystallizations were optimal for the removal of the majority of contamination, the most suitable amount of carrier dipeptide to be added allowing for recrystallization losses, was found to be 80 mg per recrystallization sequence. The recrystallization losses of synthesized labelled dipeptide and the resultant derivation of the amount of dipeptide formed in a known volume of reaction mixutre were calculated from the change in the specific activity of the dipeptide product prior and subsequent to carrier addition. Prior to carrier addition the synthesized dipeptide had the same specific activity as the parent amino acid. Upon addition of a known amount of carrier the specific activity of the total dipeptide fraction decreased and could be accurately measured upon isolation of the product. Calculation of the amount of dipeptide synthesized was derived from the following equations:

Specific acitivity of the dipeptide formed (before carrier addition)

$$= \frac{C^a}{Qf^a} = A \qquad 6:16$$

Specific acitivity of the dipeptide after dilution

$$= \frac{C^a}{Qf^a + Qa^a} = B \qquad 6:17$$

Solving for Qf^a

$$Qf^a = \frac{BQa^a}{A - B} \qquad 6:18$$

where C^a_a = number of counts in dipeptide
Qf^a = amount of dipeptide formed (mm)
Qa^a = amount of dipeptide carrier added (mm).

The specific activity of the weighed dipeptide product was determined by counting duplicate acetone samples on a Phillips automatic scintillation analyser in 5.0 ml Unisolve 1 scintillation fluid. The counting efficiency of the machine was determined for each sampled counted using an internal standard of suitably diluted n - (1-^{14}C) hexadecane, the sample counts being corrected accordingly. The counting error, i.e. the relationship between error, the magnitude of the count and the counting period, was calculated for each sample count from equation 6:19 (93) at a probability level of 95%.

$$Ex\ (\%) = \frac{100a\ \sqrt{(nb + ns)}}{ns-nb} \qquad 6:19$$

where Ex = error for net counts x (i.e. at 95% probability, in 95 cases out of 100 the count will be within ±Ex% of the recorded count).

nb = background count

ns = sample count

a = probability constant (1.96 for 95% reliable error.

Counting errors were usually within ± 5%

Scintillation effects due to the dipeptide or its acetone solvent i.e. chemiluminescence and quenching effects, were checked for by the addition of various amounts of unlabelled dipeptide to standard labelled dipeptide, with negative results.

6:6 THE SELECTION OF APPROPRIATE REACTION CONDITIONS

The first experiments were carried out in reaction systems which consisted of:

5×10^{-3} M ^{14}C CBZ glycine

5×10^{-3} M L-phenylalanine amide

3×10^{-6} M Thermolysin

in 25.0 ml 0.01 M Tris/HCl buffer, pH 8.0 with 0.01 M calcium chloride. The system was reacted for one hour at 60°C. The entire reaction mixture (from 22 ml to 24 ml) was then removed by pipette into 50 ml of ice cold acetone, containing 80 mg dipeptide carrier to precipitate the enzyme. The solution was taken down to dryness on a Buchi rotary film evaporator at 40°C and the residue dissolved in 8.0 ml boiling water, followed by a 2.0 ml water wash, filtered through a cotton wool plug and cooled in ice to induce crystallization. The precipitate was centrifuged off, washed with cold water and recrystallized from hot water with the addition of 25 mg CBZ glycine. The sample was recrystallized a total of four times, a few crystals being removed at each recrystallization stage for count/U.V. purity analysis. The dipeptide was dried in a vacuum oven at 50°C for 16 hours. The purified product was accurately weighed, a sample first being removed for a melting-point determination, and then dissolved in 2.0 ml acetone. 2 x 0.5 ml samples were counted in 5.0 ml Unisolve 1 phosphor on the liquid scintillation counter and the counts obtained processed to give the percentage of dipeptide synthesized. It was

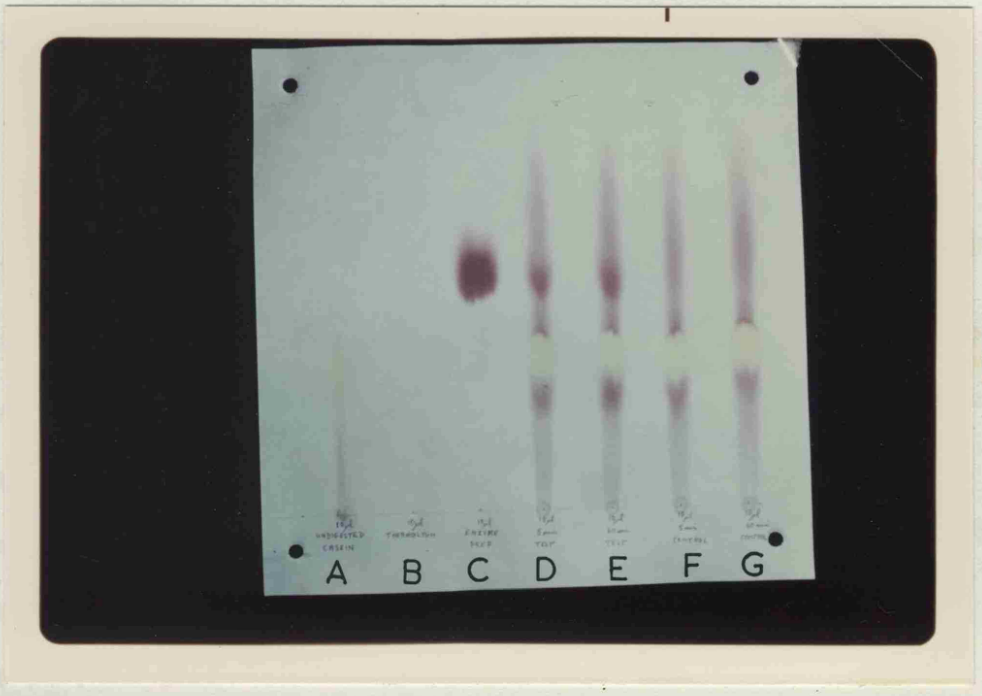
found on repeated experimentation that the amount of dipeptide synthesized varied from one reaction system to another. To test whether equilibrium had been reached two 10.0 ml samples were taken from the same reaction mixture at one and two hours. These differed in the percentage dipeptide synthesized, there being no correlation between the length of time of reaction and percent synthesis. Subsequently two one hour samples were removed from each experimental run and carried through the purification procedure. Differences in the percentage of dipeptide synthesized for the two samples were found to vary between 1% and 50% within a single experiment. The purity of the product was suspected so the recrystallization solvent was changed to methanol/water and the procedure was checked by recrystallizing unlabelled dipeptide in the presence of labelled amino acid. It was found that four recrystallization totally removed the amino acid count. Autoradiography of an electrophoretogram of the isolated dipeptide indicated that the product was essentially pure. A careful analysis of each stage of the experiment by counting aliquots of reaction mixture, products and waste liquors at every step of the procedure indicated that the difference between the duplicate samples arose between the stages of adding the reaction mixture to acetone containing carrier and the first recrystallization step. The enzyme was suspected of carrying contamination through the recrystallization procedure so the acetone/reaction mixture solution was filtered through a Millipore Fluoropore filter to

remove any precipitated enzyme. This did not remove the discrepancy between duplicate samples, suggesting that the enzyme was not precipitated or inactivated by the acetone. Since no difference in purity could be detected by thin-layer electrophoresis and auto-radiography between widely different amounts of synthesized radioactive product in duplicate samples, it was suspected that the enzyme was still active and that enzyme catalysed synthesis of the dipeptide was occurring during the evaporation of the aqueous /acetone solution on the rotary film evaporator. The rotary film evaporation stage was replaced by freeze-drying. After reaction 10 ml of mixture was added to 2.0 ml acetone containing carrier and frozen in dry ice/ethanol mixture. The samples were then freeze-dried. Duplicate samples carried through this procedure gave more consistant results, but considerable variation still occasionally occurred. In order to assess the stability of the enzyme to acetone treatment, cooling, and evaporation a non-radioactive reaction mixture was run for one hour at 60°C, a 10 ml sample pipetted into 10 ml acetone containing dipeptide carrier and the solution taken down to dryness on a rotary film evaporator at 50°C. The material was then stored on ice for two hours before being redissolved in 10 ml water. 0.5 ml of this preparation was added to 5.0 ml of a 2% solution of casein in 0.004 M NaOH, 0.03 M Tris adjusted to pH 8.0 with acetic acid, at 35°C. A control of 5.0 ml 2% casein and 0.5 ml 1 mg/ml thermolysin was also run. After 5 minutes and 1 hour aliquots were taken, cooled in ice, and 10 µl of each sample applied to Whatman No. 1 chromatography paper. The paper was developed in butanol/acetic acid/water, 48/12/20 v/v/v,

dried and visualized with 0.2% ninhydrin in ethanol. The resultant chromatogram, illustrated in figure 6:3, indicates that the enzyme remains active despite the heating, cooling, acetone precipitation and evaporating procedures. In order to prohibit further synthesis at the end of the reaction time, 2.0 ml of 0.1 M ethylenediaminetetra acetic acid (EDTA) were added to the reaction mixture to inhibit the enzyme. This measure succeeded in producing both duplicate and interexperimental agreement of results.

The experimental reaction systems finally employed to measure free energy data consisted of: 5×10^{-3} M ^{14}C CBZ glycine and 5×10^{-3} M L-phenylalanine amide in 0.01 M Tris/HCl buffer, pH 8.0 or pH 7.2, with 0.01 M calcium chloride. In later experiments an aliquot of this was removed into methanol containing carrier to provide a purification procedure blank. Thermolysin was added to the reaction mixture to a final concentration of 3×10^{-6} M or 2×10^{-8} M. 2 x 0.5 ml samples were immediately removed for subsequent volume correction counting. The system was reacted for the prerequisite time at a selected temperature before 2.0 ml of 0.1 M EDTA were added to stop the reaction. A further 2 x 0.5 ml samples were removed for volume correction counting before 2 x 10 ml samples were pipetted into methanol solutions each containing 80 mg of dipeptide carrier. The solutions were taken down to dryness on a rotary film evaporator at 50°C, the residue dissolved in the minimum of methanol and crystallization induced by cooling and the addition of water. The product was separated by centrifugation and recrystallized four times

Fig. 6:3. Chromatogram Illustrating Enzyme Stability.



A = Undigested casein. B = Enzyme.

C = Pretreated enzyme solution.

D & E = 5 min. & 60 min. incubation with pretreated enzyme.

F & G = 5 min. & 60 min. incubation with fresh enzyme.

from methanol/water with the addition of 50 mg CBZ glycine at the first recrystallization. The dipeptide products were then dried for 16 hours in a vacuum oven at 45°C. The crystals were dissolved in 1.5 ml or 2.0 ml acetone and 2 x 0.5 ml samples of each counted. Equilibrium data and free energy values were calculated from these figures.

6:7 RESULTS AND DISCUSSION

Several series of experiments were carried out each differing by a particular parameter. Series A consisted of six experiments designed to check the reproducibility of the experimental results and was carried out at 60°C. In series B the temperature of reaction was reduced to 25°C and various initial amino acid concentrations were employed, again to check the reproducibility of the results. Series C consisted of four experiments at a decreased enzyme concentration and with a different dipeptide purity assessing procedure. Due to the high concentrations of L-phenylalanine amide required to maintain the ionized L-phenylalanine amide at a level similar to that of ionized CBZ glycine, the pH of the system in series D was reduced from pH 8.0 to pH 7.2 and a complete range of reaction temperatures tried. Series E consisted of the reaction performed in both forward and reverse directions. Dipeptide hydrolysis and synthesis were carried out at various temperatures at a concentration of 1×10^{-4} M. Electrophoresis blanks were used in this last series of experiments to assess product purity.

The uncertainty interval or error quoted in the following equilibrium constant and free energy values is the sum of uncertainty intervals calculated from two sources. The sample counting error, derived from equation 6:19 was used for one error source by calculating the maximum number of extra counts associated with the counting error, adding them to the sample count, recalculating the equilibrium constant and free energy figures with this higher count and taking the difference between these values and the original values of K and ΔG^{o}, as the uncertainty interval. The second uncertainty interval was calculated from the L-phenylalanine amide ionization correction figure. The pH of the reaction system was shown to change with temperature (table 6:4), and these new pH values were found not to vary more than 0.1 pH units at a particular temperature in reaction systems measured. The pH error was derived by calculating the equilibrium constant and the free energy values using the L-phenylalanine amide correction factor appropriate to the measured pH value, (usually that in Table 6:4), and then recalculating K and ΔG^{o} with the L-phenylalanine amide correction factor at a pH value 0.1 pH units lower. The difference in these two values was taken as the second uncertainty interval. The pH uncertainty interval was only calculated for those experiments carried out at pH 7.2, $\pm$ the sum of these two uncertainty intervals gave the overall uncertainty or error for a particular calculation of the equilibrium constant and free energy change.

Series A1 to A6 Table 6:5

CBZ glycine and L-phenylalanine amide both at initial concentrations of 5×10^{-3} M and an enzyme concentration of 3×10^{-6} M. Reactions were carried out at 60°C for 1 hour at pH 8.0. Product purity was corrected for from a standard 25 cpm/0.5 ml blank. Counting errors with ± 5%.

Experiments A1 to A6 were carried out to check the reproducibility of the reaction system. The main variation in the results is due to the inadequacy of the product purity blank in compensating for the count contamination in the dipeptide product. No correction for pH change with temperature was used in the calculation of these results or in the determination of the uncertainty interval.

Series B1 to B6 Table 6:6

Various concentrations of CBZ glycine and L-phenylalanine amide at a constant enzyme concentration of 3×10^{-6} M. Experiment B6 was composed of the amino acids CBZ threonine and L-leucine amide. Reactions were carried out at 25°C for 1 hour (6 hours in the case of experiment B4) at pH 8.0. Product purity was corrected for from a standard 25 cpm/0.5 ml blank and counting errors were within ±5% except for B2 with an error of± 7.5%.

Experiments B1 to B6 were carried out at 25°C to assess the effect of concentration variation upon the equilibrium and free energy data.

Table 6:5 Experiments A1 to A6

No	Initial Amino Acid Concentration $M \times 10^3$		Enzyme Concentration $M \times 10^6$	Dipeptide Concentration $M \times 10^6$	Dipeptide/ Enzyme Mole Ratio	K_{app} litre $mole^{-1}$	ΔG^o $cal.mole^{-1}$
	CBZ gly	Phe amide					
A1	5.5	5.8	3.0	8.7	2.90	10.66 ± 3.9	-1,568±188
A2	5.1	6.5	3.0	7.4	2.45	8.69 ± 1.5	-1,453±106
A3	5.1	5.3	3.0	6.1	2.03	8.78 ± 1.4	-1,438±98
A4	5.3	5.5	3.0	10.0	3.33	13.52 ± 1.7	-1,722±82
A5	5.1	6.0	3.0	7.7	2.57	9.34 ± 2.0	-1,478±128
A6	5.6	5.6	3.0	9.3	3.10	11.85 ± 1.7	-1,632±91

Table 6:6 Experiments B1 to B6

No	Initial Amino Acid Concentration $M \times 10^3$		Enzyme Concentration $M \times 10^6$	Dipeptide Concentration $M \times 10^6$	Dipeptide/ Enzyme Mole Ratio	K_{app} litre $mole^{-1}$	ΔG^o cal. $mole^{-1}$
	CBZ gly	Phe amide					
B1	4.9	5.1	3.0	4.5	1.5	1.16 ± 0.32	-126±150
B2	2.5	2.4	3.0	1.2	0.39	1.25 ± 0.61	-132±237
B3	9.6	18	3.0	110.0	36.7	4.46 ± 0.10	-881±14
B4	4.8	7.9	3.0	5.5	1.8	0.95 ± 0.22	+ 27±125
B5	26	43	3.0	420.0	140.0	2.67 ± 0.11	-577±25
	CBZ Thr	Leu amide					
B6	5.0	8.6	3.0	21.0	7.0	1.32 ± 0.26	-149±120.

Experiments B1 and B2 agree closely in their standard free energy changes for reactions under these conditions. Experiments B3 and B5, both at increased initial amino acid concentrations, exhibit a marked decrease in ΔG^{o}, i.e. an apparent increase in the amount of dipeptide synthesized, probably due to count contamination of the dipeptide product and inadequate estimation of this impurity from the standard blank. Variation in the pH of the system at these increased amino acid concentrationsmay also have contributed to the low values. The increase in ΔG^{o} in experiment B4 is probably due to a poor estimate of the product purity combined with an effect due to a low dipeptide to enzyme ratio which became increasingly significant in later experiments. The free energy change in experiment B6 using the amino acids CBZ threonine and L-leucine amide is in close agreement with experiments B1 and B2. A pH error was not included in the uncertainty intervals.

Series C1 to C4 Table 6:7

Various concentrations of CBZ glycine and L-phenylalanine amide at an enzyme concentration of 2×10^{-8} M. Experiments C1 to C3 were carried out at 25°C, experiment C4 at 40°C, all for 18 hours at pH 8.0. Product purity was assessed for C1 and C2 using a standard 25 cpm /0.5 ml blank and for C3 and C4 using a reaction system blank, where a sample without enzyme is purified along with the experimental samples. Counting errors did not exceed ±6%.

Table 6:7 Experiments C1 to C4

No	Initial Amino Acid Concentration $M \times 10^3$		Enzyme Concentration $M \times 10^8$	Dipeptide Concentration $M \times 10^6$	Dipeptide/ Enzyme Mole Ratio	K_{app} litre $mole^{-1}$	ΔG^o cal. $mole^{-1}$
	CBZ gly	Phe Amide					
C1	5.1	7.9	2.0	7.2	360	1.19 ± 0.07	-102±36
C2	2.4	3.8	2.0	1.3	65	0.85 ± 0.20	+102±150
C3	4.1	7.7	2.0	5.5	275	1.13 ± 0.09	-73± 49
C4	5.8	9.1	2.0	14.0	700	3.74 ± 0.89	-682±179

Experiments C1 to C4 were carried out to check the reproducibility of the results at a decreased enzyme concentration and to investigate the effect of temperature. The standard free energy of peptide bond formation in experiments C1 and C3 closely agree. Experiment C2 differs from these, due, as in experiment B4, to the comparatively low dipeptide to enzyme ratio ,resulting in enzyme interference in the reaction. An inaccurate blank assessment could also have contributed to this comparatively high ΔG^{o} value. Experiment C4, carried out at $40^{o}C$, produced an expected decrease in ΔG^{o} but was exaggerated by a variation in the pH of this system with increased temperature. The uncertainty intervals were not corrected for pH.

Series D1 to D7 Table 6:8

A series of experiments at a single ionized amino acid concentration of 5×10^{-3} M, and enzyme concentration of 2×10^{-8} M. Reaction was at pH 7.2 and a range of temperatures for 18 hours. Product purity was assessed using reaction system blanks, except for D3 in which an electrophoresis blank was employed. Counting errors did not exceed ±3%. The pH of the experimental reaction systems D1, D2 and D3 was 7.2, of D4 and D5, 7.1, D6 7.0 and D7 6.9.

Series D experiments were carried out to investigate fully the effect of temperature on peptide bond formation. The reactions were carried out at pH 7.2 because of the large amounts of L-phenylalanine amide required at pH 8.0 at elevated temperatures to bring the

Table 6:8 Experiments D1 to D7

No	Temp °C	Initial Amino Acid Concentration M x 10^3 CBZ gly	Phe amide	Enzyme Concentration M x 10^8	Dipeptide Concentration M x 10^6	Dipeptide/ Enzyme Mole Ratio	K_{app} litre mol^{-1}	ΔG^o cal. $mole^{-1}$
D1	25	5.0	8.1	2.0	17	850	0.76 ± 0.10	+165±81
D2	25	5.1	11.0	2.0	19	950	0.62 ± 0.07	+282±72
D3	25	5.1	13.0	2.0	19	950	0.55 ± 0.06	+350±72
D4	40	4.9	12.0	2.0	30	1,500	0.95 ± 0.28	+ 30±98
D5	40	4.9	17.0	2.0	31	1,550	0.71 ± 0.21	+210±98
D6	60	4.9	34.0	2.0	48	2,400	0.98 ± 0.17	+ 15±127
D7	80	5.1	66.0	2.0	12	600	0.14 ± 0.04	+1,380±184

ionized L-phenylalanine amide to the same concentration as CBZ glycine. The variation in results between experiments D1 and D2 and between D4 and D5 is largely attributable to the differences in the dipeptide/enzyme ratios. The small difference in values between D2 and D3 is due to the superior electrophoresis blank employed in experiment D3 for product purity assessment. These differences are also coincidentally mirrored by the L-phenylalanine amide concentration. The anomalous result in D7 is due to the inactivation of the enzyme at 80°C before equilibrium had been reached. The uncertainty intervals include both counting error and pH error.

Series E1 to E7 Table 6:9

A series of seven experiments at a range of temperatures with initial dipeptide or amino acid concentrations of 1×10^{-4} M and an enzyme concentration of 2×10^{-8} M. Reaction was at pH 7.2 for 18 hours. The first four experiments measured the free energy change by dipeptide hydrolysis, the latter three by dipeptide synthesis. Electrophoretograms of the products were used to assess purity. Ionization correction factors based on Table 6:4 were employed. Counting errors varied from ±2% for E4, ±10% for E1 and E3 to ±25% for E5 and E6 and over ± 50% for E7.

Series E1 to E7 consisted of experiments performed at the maximum dipeptide concentration in aqueous solution of 1×10^{-4} M in both the forward (synthetic) and reverse (hydrolytic)directions

Table 6:9 Experiments E1 to E7

No	Temp °C	Initial Dipeptide Concentration M x 10^4	Enzyme Concentration M x 10^8	Final Dipeptide Concentration M x 10^6	Dipeptide/ Enzyme Mole Ratio	K_{app} litre $mole^{-1}$	ΔG^o cal. $mole^{-1}$
E1	25	1.00	2.0	0.12	6.0	23.7 ± 4.5	-1,867±114
E2	40	1.00	2.0	0.09	4.5	22.4 ± 6.2	-1,940±148
E3	60	0.99	2.0	0.14	7.0	66.4 ± 24	-2,774±182
E4	80	1.00	2.0	35.0	1,750	57,937±21,900	-7,691±158
		Initial Amino acid Concentration M x 10^4					
		CBZ gly Phe amide					
E5	25	1.00 1.0	2.0	0.08	4.0	13.5 ± 3.9	-1,530±169
E6	40	1.00 1.1	2.0	0.03	1.6	7.6 ± 5.2	-1,257±477
E7	60	1.00 1.2	2.0	0.01	0.5	4.1 ± 7.9	- 936 ±562

of reaction in an attempt to verify that equilibrium at each temperature had been reached. The increasing variation between synthetic and hydrolytic results i.e. between experiments E1 and E5, E2 and E6, and E3 and E7, is due to the combination of two factors. Very small amounts of dipeptide synthesized resulted in large counting errors attached to all the figures despite the fact that in experiments E1 to E7 the reaction mixture volumes were increased to 250 ml and 125 ml aliquots were taken for analysis. As a consequence there are large uncertainty intervals in the derived figures and as a result little relevance can be placed on experiments E6 and E7. The decreasing dipeptide/enzyme ratio also contributed to the downward trend in ΔG^{o} values in experiments E1 to E3 as opposed to the apparent upward trend in experiments E5 to E7. In experiments E5 to E7 the dipeptide concentration has not increased as expected, confirming the dubiousness of these figures. The low ΔG^{o} value in experiment E4 compared with the rest of the series, is due to inactivation of the enzyme at 80°C before equilibrium had been reached. Because of these considerable errors, comparison between the two directions of reaction throughout the temperature range is not justifiable but a tentative comparison of experiments E1 and E5 does indicate some degree of similarity which suggests equilibrium had been reached.

6:8 THE STANDARD FREE ENERGY OF PEPTIDE BOND FORMATION

Analysis of the results of Series A to E indicate that, apart from temperature, the factor that has a major influence on the

calculated values of ΔG^{o} is the dipeptide/enzyme concentration ratio.

It was an assumption of this work, based on the majority of reports into investigations on the use of reversible enzyme systems for equilibrium measurements that enzymes, in general, act as perfect catalysts (see Chapter 2), that is, they do not influence the position of equilibrium of the reaction , they merely speed its attainment. However, there is ample evidence in the results reported here that the enzyme, thermolysin, in catalysing reactions with synthetic substrates does influence the position of equilibrium. In reaction systems at pH 8.0 the difference between the free energy changes in experiments B1, B2, B6 and experiments C1 and C3, is predominantly due to differences in the dipeptide/enzyme concentration ratios. The smaller the amount of dipeptide synthesized, the smaller the ratio of dipeptide concentration to enzyme concentration and the greater the apparent free energy decrease. This bias towards synthesis at low dipeptide/enzyme ratios is further exemplified at pH 7.2 by the large difference in ΔG^{o} values between experiments E1, E5 and experiments D2 and D3. In fact the most striking evidence for enzyme interference on the equilibrium of the peptide bond forming reaction is demonstrated by comparison of Series D results with Series E results. The overall effect of the dipeptide/enzyme ratio on the equilibrium constant values is illustrated by figure 6:4. The equilibrium constants and the resultant standard free energy changes calculated from them that are least affected by the presence of enzyme are those with the highest dipeptide/enzyme ratio. As the

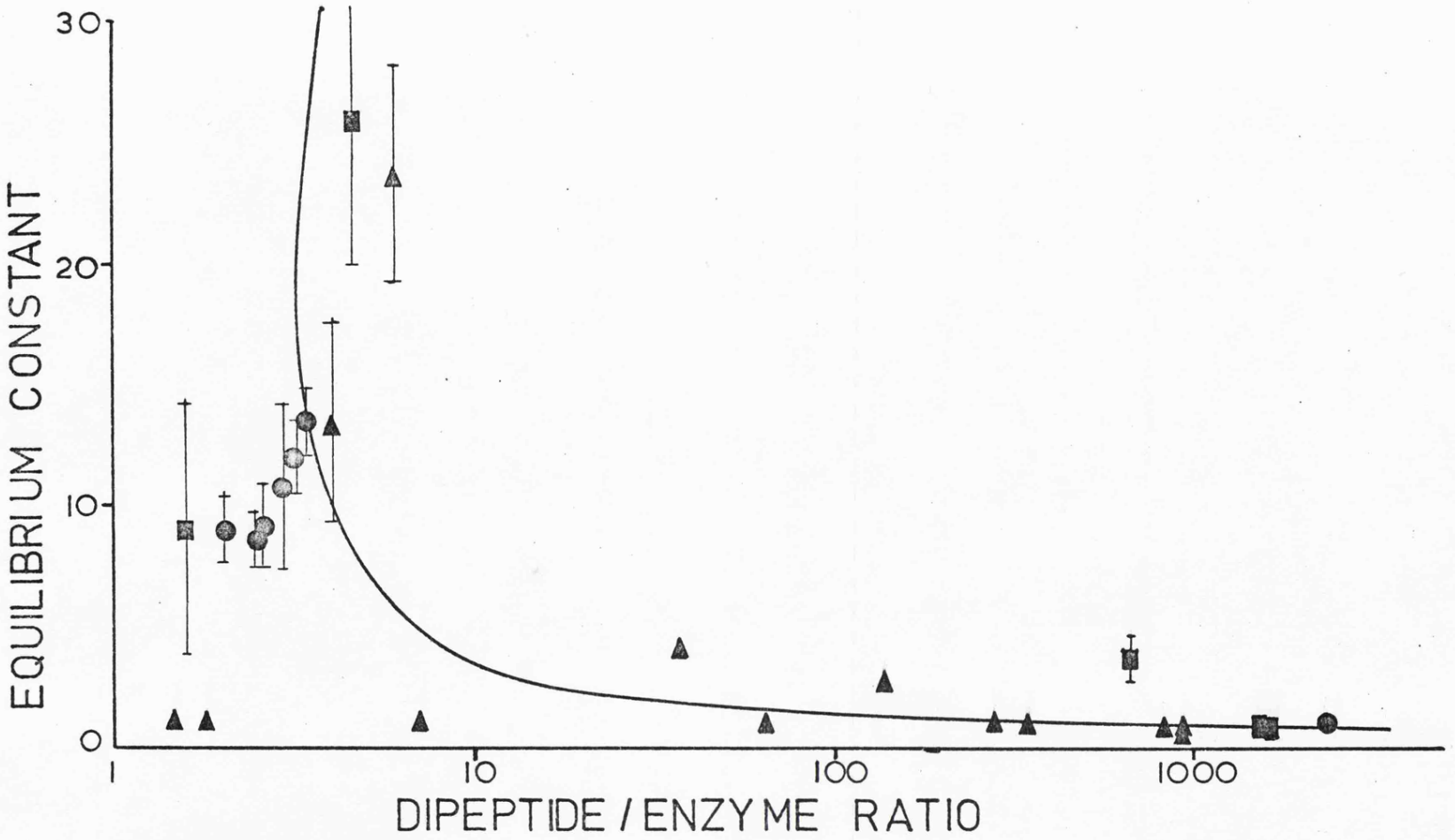


Fig. 6:4. Graph of Dipeptide/Enzyme Molar Ratio verses Equilibrium Constant.

▲ = 25°C ■ = 40°C ● = 60°C

dipeptide/enzyme ratio falls the equilibrium constant remains stable until a ratio of around 500 is reached, below which it begins to increase in value as the enzyme influences the synthesis more. Thus the most accurate equilibrium constant figures are from those reactions in which the dipeptide to enzyme concentration exceeds 500. The experiments in which the dipeptide to enzyme ratio exceeds 500 are D1, D2, D3, D4, D5 and D6. D3 is more accurate than D1 and D2 by virtue of a superior blank assessment. A higher dipeptide/enzyme ratio also favours D3 over D1 and D5 over D4. By plotting the ΔG^{o} values of D3, D5 and D6 against temperature and extrapolating the resultant straight line to temperatures of $80^{o}C$ and $100^{o}C$, the standard free energies of peptide bond formation over a complete temperature range have been presented in Table 6:10.

Table 6:10

Temperature ^{o}C	ΔG^{o} calories per mole
25	+350 ± 70
40	+210 ± 100
60	+ 15 ± 130
80	-180 (by extrapolation)
100	-370 (by extrapolation

Comparison of these free energy data with literature values for ΔG^{o} may only be effected at the lower temperatures, since such figures have not previously been reported for elevated temperatures. Reference to Table 1:1 indicates a close similarity between the results of Dobry, Fruton and Sturtevant (No. 4) and the 25°C result quoted here. The equilibrium method of ΔG^{o} estimation using isotope techniques is common to both pieces of work. It should be noted, however, that the dipeptide/enzyme ratio calculated for the synthesis of benzoyl-L-tyrosylglycinamide with chymotrypsin from the data of Dobry *et al.*, assuming pure chymotrypsin of molecular weight 25,000 and protein nitrogen 4,186, is 27. Thus the free energy figures of Dobry *et al.* are only correct if the enzyme does not affect the equilibrium of the reaction. The only other results obtained using the equilibrium measurement technique are those of Wilson and Cannan for the pyrrolidonecarboxylic acid/glutamic acid system (Table 1:1,No. 29). These measurements were carried out at temperatures of 78°C and above and vary excessively depending upon the pH of the system. Comparison of the figures derived from Wilson and Cannan's work of + 371 to - 1290 calories per mole with the result for 80°C derived in this work of - 180 calories per mole, merely indicates that the result derived here falls within that range.

The effect of temperature on the peptide bond forming reaction may be best illustrated by calculating the percentage of dipeptide

synthesized at various initial concentrations of the amino acids. This may be achieved using equation 6:20 (adapted from (2)),

$$\alpha = \left(1 - \frac{2}{1 + \sqrt{1 + 4Ka}}\right) \times 100 \qquad 6:20$$

where α, the dipeptide concentration at equilibrium divided by the initial concentration of one of the two reactant amino acids, is the percentage synthesis at equilibrium at initial concentrations of reactant amino acids, a. The two amino acids are taken to have the same concentration. K is the equilibrium constant for the peptide bond forming reaction at a particular temperature. The results are presented in Table 6:11.

Table 6:11 illustrates that at the lowest initial amino acid concentration of 0.001M, the amount of dipeptide synthesized at 100°C is three times that synthesized at 25°C. However the percentages of dipeptide present at equilibrium at both 25°C and 100°C are so small that there is very little difference in the actual amounts of dipeptide present at these two temperatures. At the highest initial amino acid concentration of 0.1 M the percentage of dipeptide present at equilibrium increases from 5% to 12.5 %, which in gravimetric terms means considerably more dipeptide at 100°C than at 25°C. For example, in a dipeptide of molecular weight 350, there would be 4.5 g of dipeptide per litre of solution at 100°C as opposed to 1.75 g per litre at 25°C.

Table 6:11

Temp ^{o}C	Initial concentrations of amino acids M	Equilibrium constant	Percent synthesis of dipeptide at equilibrium
25	0.1		4.97
	0.01	0.55	0.54
	0.001		0.055
40	0.1		6.24
	0.01	0.71	0.70
	0.001		0.071
60	0.1		8.25
	0.01	0.98	0.96
	0.001		0.098
80	0.1		10.36
	0.01	1.29	1.26
	0.001		0.129
100	0.1		12.60
	0.01	1.65	1.60
	0.001		0.164

In prebiotic terms the effect of temperature on protected amino acids (such as those adsorbed onto clay surfaces) and peptides at low concentration would be to enhance the formation of dimers, tetramers and higher polymers in dilute aqueous solution, but not increase their total concentration to any significant extent. At high initial concentrations of amino acids or peptides, arising through concentrating mechanisms such as solvent evaporation or adsorption onto a charged clay surface, there may be a considerable difference in the amount of dipeptide synthesized at 25°C and at 100°C. The actual occurrence of high concentrations of peptides or protected amino acids in aqueous solution at elevated temperatures under prebiotic conditions must be subject to debate, but, assuming such conditions did occur a plausible means of peptide bond formation, i.e. polymer production, by the reversal of hydrolysis has been presented by this work.

6:9 THE DERIVATION OF THE STANDARD ENTHALPY AND ENTROPY OF PEPTIDE BOND FORMATION

The standard heat of peptide bond formation could not be measured by the accurate technique of calorimetry due to the instability of the enzyme, especially at elevated temperatures, to the long equilibration times required in the calorimeter. As a result ΔH^{o} was calculated graphically from the equilibrium constant values using the integrated form of the van't Hoff equation:

$$\text{Ln } \frac{K_2}{K_1} = \frac{\Delta H^o (T_2 - T_1)}{T_1 T_2} \qquad 6:21$$

where K_1 and K_2 are the values of the equilibrium constant at temperatures T_1 and T_2. By plotting log K against 1/T a straight line, slope $-\frac{\Delta H}{2.303R}$ was obtained (Figure 6:5) and ΔH^o evaluated for the peptide bond forming reaction. An uncertainty interval on the ΔH^o value was determined by varying the fit of the line between the extremes of the points in figure 6:5, recalculating the slope and taking the difference in the two ΔH^o values as the maximum error, the uncertainty interval being ± half this value.

So $\underline{\Delta H^o = + 3,200 \pm 550 \text{ calories per mole.}}$

Extrapolation of the line to temperatures of 80°C and 100°C and calculation of ΔG^o from log K at these temperatures confirmed the values of -180 calories per mole at 80°C and -370 calories per mole at 100°C for peptide bond formation.

The standard entropy of peptide bond formation was derived from the equation:

$$\Delta G^o = \Delta H^o - T \Delta S^o \qquad 6:22$$

By substitution of ΔH^o and the ΔG^o value at 25°C:

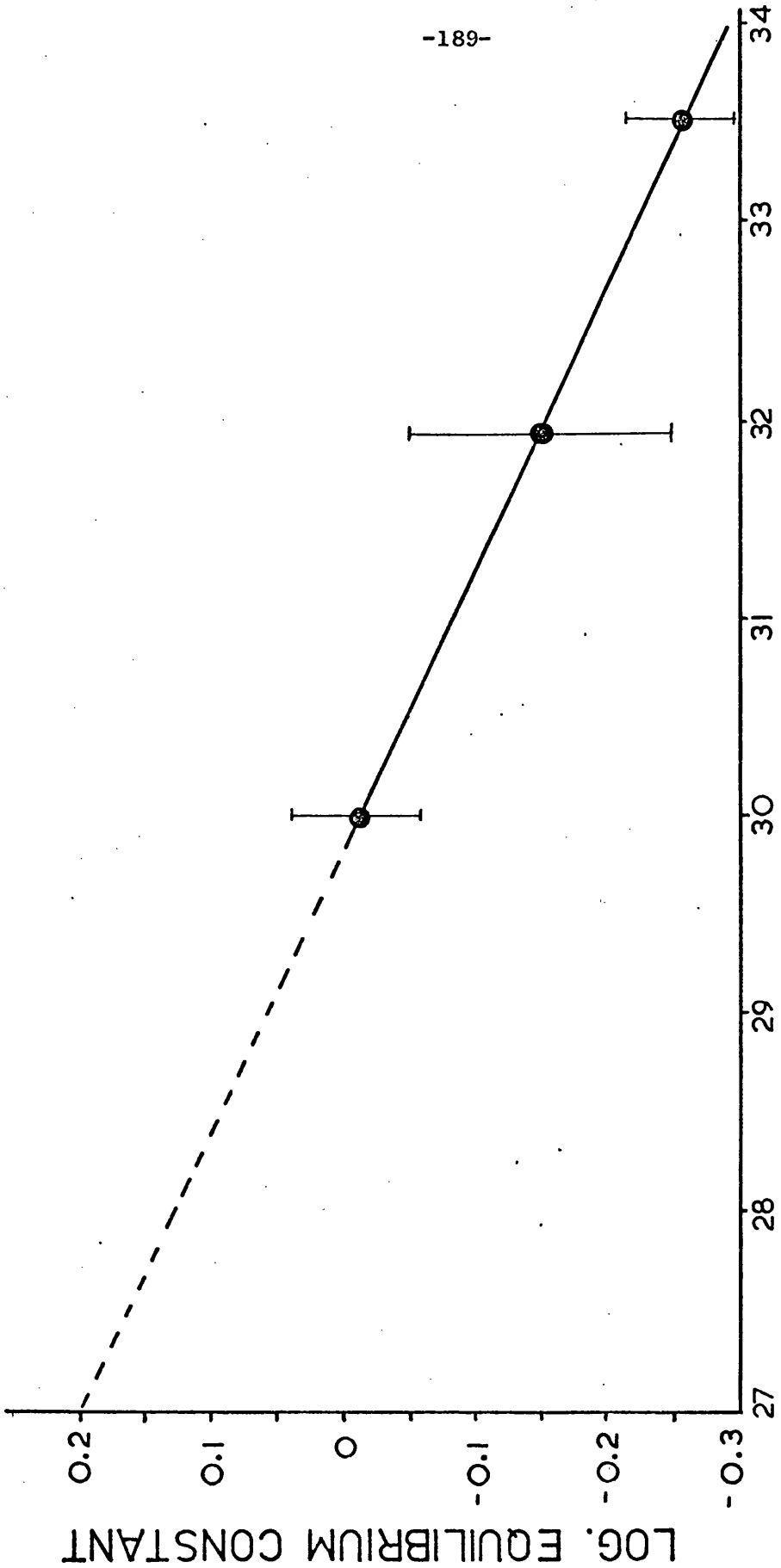


Fig. 6:5. Plot of Log. K verses 1/T for the Determination of H^{o}.

ΔS^{o} = +9.6 ±2.0 calories per mole per degree

The error was calculated from the uncertainty interval of ΔH^{o}.

The enthalpy change derived from the equilibrium measurements at elevated temperatures of +3,200 calories per mole compares favourably with the values of Wilson and Cannan for pyrrolidonecarboxylic acid of +3,600 and +3,800 calories per mole (Table 1:1, No. 29), which were similarly calculated from the van't Hoff equation, but is generally higher than the majority of values obtained by reaction calorimetry, reflecting the inaccuracies in the method chosen to determine it. Nevertheless the enthalpy value and the entropy value, which also agrees with data derived from the results of Wilson and Cannan as well as combustion calorimetry data, but again is higher than Dobry's value because of a higher ΔH^{o} value, emphasize the relatively low thermodynamic energy changes occurring during the formation of an internal peptide bond.

6:10 CONCLUSIONS

The free energies of peptide bond formation derived from equilibrium measurements are:

at 25°C + 350 ± 70 calories per mole

at 40°C + 210 ±100 calories per mole

at 60°C + 15 ±130 calories per mole.

and by extrapolation are:

at 80°C - 180 calories per mole

and at 100°C - 370 calories per mole

The average decrease in the free energy with rising temperature over the range investigated is only 9.6 calories per mole per degree. At low initial amino acid concentrations at high temperatures the yield of dipeptide is not substantially greater than at 25°C. At high initial amino acid concentrations there is a significant increase in the amount of dipeptide synthesized at elevated temperatures as opposed to a temperature of 25°C.

Peptide bond synthesis by the reversal of hydrolysis is considered a possible polymerization mechanism under prebiotic conditions.

The enthalpy of peptide bond formation derived graphically from the van't Hoff equation is:

+3,200 ± 550 calories per mole

The entropy of peptide bond formation is:

+9.6 ± 2.0 calories per mole per degree.

The enthalpy and entropy results are higher than expected due to inadequacies in the method used to estimate ΔH^{o}.

There is strong evidence for the involvement of the enzyme, thermolysin, in the reaction equilibrium at low dipeptide to enzyme ratios.

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APPENDIX

Abbreviations:

Lys.	Lysine/lysyl
Bzl.	Benzoyl
Tyr.	Tyrosine/tyrosyl
Gly.	Glycine/glycyl
NH_2	Amide
CBZ	Carbobenzoxy or benzyloxycarbonyl
Leu.	Leucine/leucyl
Phe.	Phenylalanine/phenylalanyl
Ala.	Alanine/alanyl
Hipp.	Hippuric acid/hippuryl
An.	Aniline
HAc.	Acetic Acid
Phz.	Phenylhydrazine
$BuNH_2$	Butylamine
Pyrol.	Pyrrolidonecarboxylic acid
Glu.	Glutamic acid/glutamyl
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
PCMB	p-Chloromercuribenzoate